

PANCREATIC SECRETORY TRYPSIN INHIBITOR

experimental and clinical studies

in the rat and man

William H. Marks



Lund 1983

Malmö 1983



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to Chris, A.J., & D.W.

THIS DISSERTATION IS BASED ON THE FOLLOWING WORKS: *

- I Isolation and partial characterization of the pancreatic secretory trypsin inhibitor in the rat.
Marks WH, Ohlsson K.
Biochim. Biophys. Acta 717:91-97, 1982.
- II A radioimmunoassay for rat pancreatic secretory trypsin inhibitor.
Marks WH, Ohlsson K.
Scand. J. Gastroenterol. 18:000-000, 1983.
- III Pancreatic secretory proteins in sera from rats before and after induction of experimental pancreatitis.
Marks WH, Genell S, Hjelmqvist B, Ohlsson K.
Accepted, Scand. J. Gastroenterol.
- IV Studies on the interaction of Trasylol (aprotinin) with trypsin-pancreatic secretory trypsin inhibitor (PSTI) complexes and with α_2 -macroglobulin-trypsin-PSTI-complexes.
Marks WH, Eddeland A, Ohlsson K.
Hoppe-Seyler Z. Physiol. Chem 362:1653-1656, 1981.
- V On the role of the pancreatic secretory trypsin inhibitor as an inactivator of trypsin- α_2 -macroglobulin complexes in acute pancreatitis.
Balldin G, Borgström A, Marks WH, Ohlsson K.
Submitted J. Clin. Invest. 1983.
- VI The elimination of pancreatic secretory trypsin inhibitor from the circulation. A study in man.
Marks WH, Ohlsson K.
In press, Scand. J. Gastroenterol. 1983
- VII Endoscopic collection of pure pancreas juice for experimental analysis: a simple protocol for routine use.
Marks WH, Wehlin L, Ohlsson K, Russick C.
Submitted, J. Surg. Endoscop. 1983.
- VIII Pancreatic secretory trypsin inhibitor, cathodal trypsin and pancreatic elastase in pancreas juice collected at ERCP.
Marks WH, Ohlsson K, Wehlin L, Russick C.
Submitted, Surgery 1983.
- IX Immunocytochemical distribution of trypsinogen and pancreatic secretory trypsin inhibitor (PSTI) in normal and neoplastic tissues in man.
Marks WH, Ohlsson K, Polling Å.
Submitted, Scand. J. Gastroenterol. 1983.

* These works will be referred to by their Roman numerals throughout the thesis.

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I. INTRODUCTION

The human exocrine pancreas produces about 1.5 liters of juice per day, containing approximately 25 g/l of protein. It has the highest turnover of protein per gram weight of any organ in the body. These protein products are made up of about 25 to 30 components as demonstrated by ourselves and others using isoelectric focusing (25,60,87). Many of these components have been described chemically and several have redundant functions (38,76). These proteins are predominantly digestive enzymes having amylolytic, lipolytic or proteolytic activity. Most of the proteolytic enzymes are secreted in their inactive or zymogen forms and are normally activated in the duodenum by trypsin following its own activation by the duodenal brush border enzyme, enterokinase.

A central role for trypsin in the normal physiology of the exocrine pancreatic digestive system is accepted. However, its role in the pathogenesis of acute and chronic pancreatic diseases remains unclear (43,76).

The potentially catastrophic consequences of prematurely activated proteases were recognized early-on by such authors as Chiari (29) and Opie who suggested a pathogenic role for activated trypsin in the pancreas gland (73).

Laskowski Jr. has suggested (63), and others are continuing to confirm, that tissues which contain proteolytic enzymes also possess enzyme inhibitors. These enzyme inhibitors make up a 'system' of low molecular weight (LMW), locally-produced protease inhibitors. Examples have now been demonstrated in semen (89), the cervix (26,98), broncho-pulmonary system (49,50), ear (22), and the pancreas (59), and an imbalance in the local protease-anti-protease equilibrium appears to play a role in the pathology of the specific organ in question (8).

In 1948 Kazal described an acid-stable trypsin-specific inhibitor in bovine pancreas extracts (59). Kazal described it as a secretory protein of the exocrine pancreas and it is now known as the Pancreatic Secretory Trypsin Inhibitor (PSTI). PSTI has been identified in both tissue extracts and pure pancreas juice.

Results from cell-fractionation studies indicate that it is produced by the acinar cells (7), although this has to our knowledge not been proven by direct immunohistologic data. Analogous inhibitors have been identified in the dog (39,48), rat (56), human (54, 58,80), cow (55), and pig (53). Pubols, *et al* have done extensive work on the chemistry of the human inhibitor. In 1974 they isolated 5 chromatographic forms of the molecule having identical molecular weights, amino acid compositions and specific activities (80). In 1976 Barrett, *et al* published the amino acid sequence of the human inhibitor (11). Eddeland and Ohlsson then published a series of works which included the development of specific immunochemical assays for both human and canine PSTI. Using these immunoassays they quantitatively detailed the behavior of PSTI in the pancreas juice and serum of normal dogs and human volunteers, and also during acute and experimental pancreatitis.

In man, PSTI is present in about .1 to .8% (38,39,46,53,55) of the total protein concentration of the pancreas juice, corresponding to approximately 2% of the cationic trypsin production. Five chromatographic forms are immunologically identical (38). Eddeland clearly demonstrated that in normal serum and pancreas juice, PSTI exists in its free and uncomplexed form. He also showed that in serum type milieus PSTI-trypsin complexes were readily cleaved and trypsin was given up primarily to the major serum protease inhibitors, α_2 -macroglobulin (α_2 -M) and α_1 -anti-trypsin (α_1 -AT) (36,40).

When PSTI is released from a complex with trypsin it is probably altered. Laskowski Jr. has previously demonstrated that PSTI undergoes a series of internal hydrolyses following complexation with trypsin (64). If PSTI is cleaved from trypsin following the first hydrolysis, it is still active, but apparently to a lesser degree than that of native PSTI. If the trypsin inhibitor complex is left uncleaved the process continues and eventually results in the release of inactive fragments of PSTI (97). For this reason PSTI has been classified as a "temporary inhibitor".

As noted above, in serum type milieus PSTI plays a less important role in trypsin inactivation than either α_2 -M or α_1 -AT. This strongly supports the concept that PSTI serves an escort function,

i.e. it complexes prematurely activated trypsin and escorts it to a safe area such as the gut or the circulation where it can be given up to α_2 -M for elimination (36,40,41). Therefore, PSTI appears to have little if any role as a systemic trypsin inhibitor. However, it has been demonstrated that α_2 -M-trypsin complexes continue to be active against low and some high molecular weight substrates. *In vitro* α_2 -M-trypsin complexes are capable of activating trypsinogen at 50% the rate of unbound trypsin (21,85). This is probably due to the mechanism of action of α_2 -M which acts as a giant 'bear trap', enveloping active serine proteases, but not necessarily directly blocking their active site. It protects most high molecular weight (HMW) substrates from active trypsin by two mechanisms. The first is directly by steric exclusion of the substrate from the active protease. The second is indirect and more subtle, but probably no less important. The reticuloendothelial system (RES) of the liver recognizes activated α_2 -M-protease complexes and rapidly eliminates them ($T_{1/2}$ of 10 min) from the circulation (75). However, in more static milieus, i.e. those of inflamed tissues such as the abdominal cavity in acute pancreatitis, the elimination of the protease- α_2 -M-complexes is probably slower. Under these circumstances α_2 -M-protease complexes might perform a destructive function by activating potentially damaging substances such as other proteases like trypsinogen (21,85) or by destroying substances like the parathyroid hormone (23). Teleologically, it would seem ideal if LMW active site inhibitors such as PSTI were capable of inhibiting these complexes.

Studies to date concerning the behavior or role of PSTI in normal and pathological situations have been limited. As noted above, Eddeland pioneered the study of immunoreactive PSTI in the dog and man, in normals and during acute pancreatitis. More recently, Ogawa, *et al* have described the behavior of PSTI in the circulation of patients having acute pancreatitis also using a conventional radioimmunoassay (61,72). Both investigators noted marked increases in circulating levels of PSTI during acute pancreatitis, however, in Ogawa's study levels were elevated to such a degree that PSTI served as a better indicator of acute pancreatitis than did either trypsin or α -amylase. Clearly, more

detailed clinical and experimental studies would be useful to characterize the behavior of PSTI during acute pancreatitis, as well as to establish its diagnostic potential in this disease. Studies of PSTI in man with diseases other than acute pancreatitis are also desirable. Previous authors have noted decreases in the "enzyme" content of pancreas juice in patients with chronic pancreatitis and pancreatic carcinoma (3,27,31,34). However, "protein" has been equated with "enzyme" in most works and only one study, to our knowledge, currently exists which demonstrates the behavior of exocrine pancreatic proteins other than enzymes in disease processes of the pancreatico-biliary system (86). The study of PSTI in conjunction with some known exocrine pancreatic products may prove useful in elucidating normal and pathological physiology of the pancreas.

II. PURPOSE OF THE PRESENT INVESTIGATION

The primary aim of this work was to determine what role PSTI plays in pathological states of the pancreas. Special interest was focused on the interaction of PSTI with trypsin and trypsin- α_2 -macroglobulin complexes *in vitro* and *in vivo* in man and rat.

To facilitate the experimental studies of PSTI in the rat it was necessary to isolate, characterize and develop an immunoassay for the protein as this had not been done previously.

The following special questions were asked:

- 1) Can PSTI inhibit the activity of α_2 -M-bound trypsin in a biologically important way?
- 2) Can PSTI be displaced from trypsin and/or α_2 -M-bound trypsin by another LMW inhibitor, aprotinin?
- 3) How does PSTI behave in the peritoneal exudates of patients with acute pancreatitis?
- 4) How does pancreatic disease affect the concentration of PSTI in the pancreas juice?
- 5) What is the elimination pattern of PSTI from man?
- 6) Where is PSTI distributed in the pancreas gland with respect to trypsin?

III. PRESENT INVESTIGATION

A. Isolation of human and rat PSTI, and partial characterization of rat PSTI (I)

Human PSTI has previously been isolated by several authors using different isolation protocols (38,80). Eddeland used lyophilized pancreas juice which was dissolved in a small amount of NaAc buffer (pH 4.5) and then gel filtrated as his initial isolation step. The intent of his protocol was to preserve other proteins as well as PSTI. In order to protect against activated trypsin in the sample soy trypsin inhibitor was added to his initial charge. We also wished to isolate PSTI from both human and rat pancreas juice and likewise preserve other proteins such as trypsin for later use. To accomplish this without the addition of an exogenous trypsin inhibitor, we used a modification of Eddeland and Ohlssons' scheme.

In our procedure lyophilized pancreas juice was re-dissolved in 10^{-2} M HCl under constant stirring and pH monitoring. Concentrated HCl was then cautiously added by dripping down the side of the beaker until pH 2.5 was reached. At this pH, trypsin activity is essentially nil and trypsin cannot associate with PSTI. This decrease in pH resulted in the creation of a large precipitate which was centrifuged off and washed twice with 10^{-2} M HCl. After combining these washes with the original solution, the mixture was added to a Sepharose G-50 column for initial chromatographic separation.

1. Human PSTI:

Fractions eluting from this Sephadex G-50 column were monitored using rabbit anti-PSTI (38) and sheep anti-human cationic trypsin (20) which are available in our laboratory. Further purification was performed as previously described (38,39) using ion exchange and desalting chromatography and ultrafiltration followed by lyophilization.

Trypsin is a highly immunogenic molecule which requires no special manipulations to produce antiserum. However, PSTI is a very poor antigen. Greene used albumin conjugated PSTI in order to make an anti-serum to bovine PSTI (46). We were able to enhance the immunogenicity of PSTI by briefly exposing it to trypsin prior to inoculation.

To facilitate this, native PSTI was placed on a CnBr-Sepharose 4B bovine trypsin affinity column at pH 7.4. Following a one column volume wash with the buffer, PSTI was eluted as a single peak with .01 M formic acid solution (pH 2.5). Goat anti-human PSTI was then prepared using 1.0 mg of bovine trypsin-exposed PSTI in Freund's complete adjuvant, followed by 3-4 weekly booster doses of 500 µg. Previous authors have described serial hydrolysis of analogous molecules from other species in the presence of trypsin. The initial hydrolysis of PSTI results in an opening up of this tightly packed molecule which retains its antitrypsin properties (64,90,97). It seems likely that this opening up of the PSTI molecule increases accessibility of its immunogenic sites. Specific rabbit anti-human cationic trypsin was prepared using 500 µg of the pure antigen in Freund's complete adjuvant, followed by booster doses of 250 µg every three weeks.

2. Rat PSTI and anionic trypsin:

Pure rat pancreas juice was collected using a method described by Genell (52). Following induction of anesthesia with intraperitoneal chloralhydrate a small upper midline incision was made. The distal hepato-pancreatic duct was cannulated with a PE-10 catheter and the proximal duct ligated in order to exclude bile. The catheter was brought out through the tail and the animal secured in a specially designed cage. Animals were allowed to drink and feed *ad libitum* and kept alive for a maximum of 4 full post-op days. Juice was collected in beakers on dry ice. Each animal yielded 10 to 20 ml/24 hrs although juice flow was poor until 24 hrs post-operatively.

Anionic trypsin, the predominant form of trypsin in rats, was isolated as described by Genell *et al* (52). Trypsin activity was monitored using the chromogenic low molecular weight substrate BAPNA (Benzoyl-L-arginine hydrochloride) according to Erlanger (44).

The isolation of rat PSTI was accomplished using a method similar to that employed for human PSTI. PSTI was monitored by its ability to inhibit bovine trypsin activity on BAPNA.

Rat PSTI, although grossly identified in pancreas tissue extracts by Grossman in 1958 (56), has not previously been isolated or characterized. Our isolation technique resulted in a 56% yield, about the same as others have reported for PSTI from different species (38,39,53,80).

Rat PSTI was present in whole pancreas juice in approximately .2% of the total protein content. This is a similar proportion as reported for other species (38,39,59). Two distinct chromatographic forms were present on polyacrylamide disc electrophoresis at pH 4.5. Five bands were present on standard slit agarose gel electrophoresis. Unlike the PSTI of other species, which are anodal, rat PSTI is cathodal in a basic barbital buffer at pH 8.2. Rat PSTI is immunologically distinct from human PSTI and is trypsin-specific, inhibiting active site titrated rat trypsin (28) in a 1:1 molar ratio (I). It is a good inhibitor of bovine trypsin, a poor inhibitor of human cathodal trypsin, and did not inhibit rat chymotrypsin (I). Antiserum was manufactured in the rabbit using the techniques detailed above for human PSTI.

B. Radioimmunoassay (RIA) for rat PSTI and its behavior during experimental pancreatitis (II, III)

Many models have been developed for the study of acute pancreatitis. Rat models are generally simple and inexpensive. Arnesjö (6) and Evander (45) have described a model using direct taurocholate and/or trypsin infusion into the pancreatico-biliary duct. Aho and Nevalainen followed electron and light microscopic changes in rat pancreas following duct injection of plain buffer and buffer containing the bile salt, taurocholate (TC). They found evidence that pancreatitis developed in two stages. The initial lesion seemed to result from the pressure of the injection. They theorized the action of TC on cell membranes then resulted in a second and more pronounced stage which was attributed to protease activity (1,2).

To date only one report involving circulating levels of pancreatic proteins other than amylase in the rat during acute pancreatitis has been published. Satake *et al* followed pancreatic elastase

immunoreactivity and α -amylase enzymatic activity in the peripheral circulation of rats during acute pancreatitis induced by pancreatic duct injections of buffered saline with and without active trypsin (88). We speculated that the behavior of PSTI in the peripheral circulation during acute pancreatitis could provide clues as to its role in the diseased gland. Therefore, we chose to study the behavior of PSTI in a similar model to that of Aho and Nevalainen. Anionic trypsin and α -amylase were also studied.

Trypsin was excluded from the infusate, relying only on TC to induce pancreatitis. This was done in order to avoid complexation of endogenous PSTI by exogenous trypsin. We also hoped to avoid the contributions of exogenous trypsin activity to the induction of the pancreatitis. In order to study circulating levels of PSTI and anionic trypsin, which is the predominant trypsin in rats, it was necessary to develop radioimmunoassays for them.

1. Radioimmunoassay for PSTI and anionic trypsin:

In each case purified antigen was used to generate a specific antiserum. Specificity was determined by immunoelectrophoresis and Ouchterlony double immunodiffusion. Antigen was labeled with ^{125}I using a lactoperoxidase technique as described by Thorell (95).

We were able to precipitate about 90% of each labeled pure antigen with the appropriate antiserum. Approximately 7 μg of each antigen was tested against a 1:2 dilution series of the appropriate antiserum. For anti-PSTI the 60% titer was 1:10,000 and for anti-trypsin 1:100,000.

Dilution curves for standard PSTI and PSTI in serum and pancreas juice were parallel. For anionic trypsin-like immunoreactivity similar curves were generated for serum, pancreas juice and feces extracts (II). The serum and pancreas juice curves were parallel and the standard curve and that of the fecal extract were very close to parallel. DFP inactivated trypsin was used for the standard trypsin antigen and thus strictly parallel curves indicating identity may not necessarily be anticipated. Similar results were described for human cationic trypsinogen and DFP inactivated cationic trypsin standard by Borgström (19,20). However, Satake *et al* demonstrated parallel dilution curves for another serine

protease, pancreatic elastase, and DFP-pancreatic elastase (88). RIA analysis of gel filtrated serum and pancreas juice revealed a solitary trypsin peak and a solitary PSTI peak. These results parallel those in man, indicating PSTI in rat normally exists in its free state. Further, PSTI was eluted as a single peak in an identical volume as were the low molecular weight basic protease inhibitor Aprotinin ($mw \approx 6300$) and human PSTI ($mw \approx 6200$).

The normal circulating level of PSTI in fasting Wistar rats ($n=20$) was $15.0 (\pm 4) \mu\text{g/l}$. In the same rats the anionic trypsin concentration was $126.0 (\pm 18) \mu\text{g/l}$. Minimal detectable levels of PSTI and anionic trypsin were $0.75 \mu\text{g/l}$ and $6.0 \mu\text{g/l}$ respectively.

2. Pancreatitis:

Pancreatitis was induced in ten Wistar rats weighing 190 to 220 g. Following adequate intraperitoneal chloralhydrate anesthesia, the abdomen was entered through an upper midline incision. The duodenum and pancreas were delivered and the hepato-pancreatic duct identified. The proximal duct was occluded with a vascular clamp and a PE-50 polyethylene catheter was placed transduodenally into the ampulla. This was held in place with a second vascular clamp. A buffered solution containing 4% TC was infused at $400 \mu\text{l}$ over 75 seconds using a Harvard pump. Blood samples were taken pre-infusion (baseline), and at 2, 15, 30, 60, 120 and 360 min. Five other rats acted as 'controls' and were handled identically except that TC was omitted from their duct infusate.

At necropsy animals infused with buffer and 4% TC had sero-sanguinous fluid in the peritoneal cavity and major inflammatory changes with gross areas of hemorrhage were apparent. Those animals receiving buffer alone had some areas of edema but no other obvious changes or fluid in the abdomen.

Graphic analysis of the serum levels of α -amylase and immunoreactive trypsin in the TC group revealed an initial sharp rise followed in 30 to 60 minutes by a second marked rise which continued throughout the experiment. PSTI also rose sharply but no plateau was present, i.e. PSTI levels also continued to increase sharply throughout the experiment.

In the non-TC group there was a sharp initial rise for all 3 proteins. This was followed by a prolonged fall back to baseline levels for PSTI and α -amylase within 6 hours. Immunoreactive trypsin remained elevated throughout the experiment and at 6 hours was still 150% over the baseline value.

The shapes of the curves in the TC group are in agreement with the findings of Aho and Nevalainen (1,2) and support their proposal that there are two phases of the disease in this model. Phase one being due to the pressure of the injection forcing reflux of pancreatic proteins to the circulation and causing some acinar disruption. This may be likened to the findings of previous authors who described benign hyperamylasemia, trypsinemia and increases in circulating PSTI following ERCP in normal humans (42,91).

The second phase probably results from the activity of proteases following the action of TC on the cell membranes. The delay in the onset of this second phase may be due to the time necessary for TC to work on the cell membranes and induce protease activation. It may also represent the time needed for the activated trypsin to overwhelm the available PSTI.

Gel filtration of normal rat serum resulted in immunoreactive trypsin and PSTI each eluting as single peaks in a volume identical to that of free trypsin and PSTI, respectively. Similar experiments using serum from rats with acute pancreatitis resulted in trypsin immunoreactivity eluting in a volume consistent with free trypsin and in addition in a volume consistent with that of the serum protease inhibitors indicating complexation of active trypsin by the serum protease inhibitors. In addition a large amount of trypsin immunoreactivity was eluted in a broad peak coming after the volume of free trypsin indicating immunogenic degradation products of the enzyme.

PSTI immunoreactivity eluted in 2 peaks. The smaller of the two eluted at the volume of the free inhibitor and the larger at a volume coming just before the elution volume of the column. This too indicates that during acute pancreatitis breakdown products of the inhibitor containing immunogenic sites are present in the circulation.

This observation supports the assumption that trypsinogen is activated in this form of pancreatitis as contact with active trypsin is known to result in the hydrolysis of the inhibitor (64,97).

C. Interaction between human PSTI-trypsin and human PSTI-trypsin- α_2 -macroglobulin complexes and Trasylol, and behavior of PSTI in the serum and peritoneal exudates of patients with acute pancreatitis (IV, V)

Attempts to attack pancreatitis at a pathophysiologic level by inhibiting active trypsin with the exogenous inhibitor, Trasylol, have been met with mixed results (10,69,96). PSTI (mw=6200) and Trasylol (mw=6300) are both LMW trypsin inhibitors which work in a 1:1 molar ratio to inhibit trypsin.

As noted in the introduction, it can be argued that in and immediately around the inflamed gland, trypsin-PSTI complexes could exist at least until serum exudates appear. With serum come α_1 -AT and α_2 -M. The vast majority of trypsin is given up by PSTI to these serum protease inhibitors. If severe or prolonged inflammation exists and a static environment is formed, i.e. without free inflow and outflow of blood, then local α_2 -M and α_1 -AT could become saturated and trypsin-PSTI complexes would eventually deteriorate as a phenomenon of 'temporary inhibition' leaving active trypsin (90,97). As Trasylol is not a temporary inhibitor it would be preferable to have trypsin-Trasylol complexes. Administration of Trasylol could then theoretically result in stably inhibited free trypsin. Likewise, it might result in the incorporation of Trasylol into α_2 -M-bound trypsin complexes, thus also inhibiting these partially active complexes (12,41,51).

When PSTI-trypsin complexes were made and analyzed for PSTI, using either RIA or radial immunodiffusion according to Mancini no PSTI was detectable (66). When the complexes were incubated and gel filtrated at acid pH our anti-PSTI formed precipitates with fractions eluted in the volume corresponding to free PSTI. Therefore, we concluded that our rabbit anti-PSTI antiserum could detect only free PSTI. Next we created trypsin-PSTI complexes by incubating human trypsin with an excess of PSTI and then gel filtrating the mixture. Aliquots of these complexes were incubated with increasing

amounts of Trasylol. The samples were then subjected to radial-immunodiffusion using rabbit anti-human PSTI. After incubation of PSTI-trypsin complexes with Trasylol, immunoprecipitates appeared showing that the PSTI trypsin complexes are dissociated and that PSTI is readily displaced by Trasylol from its complex with trypsin (IV). This indicates that Trasylol should be able to fulfill the function of the plasma protease inhibitors by stably inhibiting initially PSTI-bound trypsin in acute pancreatitis.

α_2 -M-trypsin-PSTI- ^{125}I complexes were then manufactured according to Eddeland (41). Trasylol was added in increasing molar excess to the freshly prepared complexes. However, in this situation only 20% of the available counts could be released even with a ten-fold molar excess of Trasylol. The displaced activity was gel filtrated and appeared in 2 peaks. The major peak eluted in the volume of free PSTI, the second in a volume just preceding the column volume suggesting degradation products of PSTI which still carried radio-label (IV).

Next we titrated complexes of human trypsin and human α_2 -M with human PSTI using trypsin- α_2 -M complexes of varying trypsin contents in an attempt to elucidate the role of PSTI as a biologically important inhibitor of these complexes. The results of these *in vitro* studies were then correlated with our findings from direct analysis of immunoreactive PSTI in the plasma and peritoneal exudates from patients with acute pancreatitis.

α_2 -M-trypsin complex inhibition by PSTI

Complexes of human α_2 -M and trypsin were manufactured in two batches. The first was made as saturated α_2 -M-trypsin complexes and the second as half saturated complexes.

PSTI blocked the activity of the trypsin- α_2 -M complexes in a slow dose-dependent reaction. The semi-saturated complexes were inhibited more readily than the saturated complexes. Equilibrium was reached within 60 minutes for both complexes but the inhibition was not complete even with large amounts of PSTI. This is in

accordance with the results

2. PSTI in peritoneal fluid of patients having acute pancreatitis:
Serum and peritoneal exudates were analyzed for their content of PSTI in patients having acute pancreatitis. Mean levels of 155 μ g/l and 180 μ g/l were found, respectively.

Exudates (5 ml) were charged to a Sephadex G-100 column using a .1 M Tris-HCl buffer at pH 7.4 and containing .5 M NaCl and 0.005 M EDTA.

α_2 -M and α_1 -AT both eluted as single peaks while immunoreactive trypsin eluted as two peaks corresponding to trypsinogen and trypsin- α_1 -AT complexes. Immunoreactive PSTI regularly eluted as a single rather broad peak in a volume roughly corresponding to the size of the free inhibitor. Degradation products of PSTI may, however, be included in this peak. The serum levels of PSTI were in agreement with earlier data from our lab (37) but were considerably lower than those recently reported from Japan (61,72).

The α_2 -M containing fractions were pooled and concentrated to 2 ml by ultrafiltration. 1 ml of this charge was incubated in 1 ml of the original buffer, and 1 ml was incubated with 1 ml of .1 M formic acid pH 2.5. At this pH there is a disruption of the α_2 -M protease complex. These samples were then gel filtrated on a Sephadex G-100 column. Fractions were analyzed for cathodal trypsin and PSTI using RIA. No PSTI immunoreactivity was present despite the demonstration of PSTI immunoreactivity in the fluid to levels of 400 μ g/l. On the other hand, trypsin was always present.

We felt, however, that the inability to demonstrate PSTI in the α_2 -M-trypsin complexes might depend on the methodology. Saturated and half-saturated trypsin- α_2 -M complexes were therefore titrated *in vitro* with PSTI to equilibrium. The PSTI-trypsin- α_2 -M complexes were then past through a Sephadex G-200 column to separate unbound PSTI. When these *in vitro* prepared PSTI-trypsin- α_2 -M complexes were analyzed according to the same methodology as the *in vivo* complexes, trypsin as well as PSTI were both regularly demonstrable.

Taken together, the data presented in Section 1 and 2 strongly argue against any biological significance of PSTI as an inhibitor of trypsin- α_2 -M complexes. Biologically important enzyme-inhibitor reactions reach equilibrium within fractions of a second not of

an hour.

Trypsin- α_2 -M complexes were regularly found in the peritoneal exudates of patients with severe acute pancreatitis and also free PSTI, but no immunoreactive PSTI could be identified in the α_2 -M complexes. This is also in agreement with earlier findings demonstrating considerable trypsin-like activity in the macroglobulin fraction from peritoneal exudates in experimental (74) and severe acute pancreatitis (24).

D. Elimination of PSTI from the circulation in the human (VI)

Patients having acute pancreatitis had markedly elevated levels of PSTI. This was to be anticipated assuming PSTI would follow the pattern of other pancreatic exocrine proteins. Ogawa, *et al* recently reported a clinical study in which circulating levels of PSTI in patients having acute pancreatitis were massively elevated (61,72). In their study mean PSTI levels were elevated 75 times baseline as compared to about 15 times baseline in our study. More importantly, they reported that PSTI served as a better indicator of acute pancreatitis especially later in the disease, than did either α -amylase or trypsin. This finding represented a potentially useful clinical method.

Previous authors studying the elimination of similar molecules in lower animals demonstrated a rapid disappearance from the peripheral circulation with the simultaneous appearance of whole molecules or degradation products in the urine. This makes sense as molecules under 50,000 Daltons tend to be rapidly eliminated by the kidneys (93). Therefore, in light of Ogawa's report, we felt that a study of the elimination of free PSTI from the circulation in man was warranted. Increased plasma levels of PSTI late in acute pancreatitis indicate there is a prolonged leakage of PSTI into the serum or a delayed clearance of PSTI from the serum.

We labeled 250 μ g of human PSTI with 4.25 MBq 125 I using a lactoperoxidase method (96). Gel filtration on Sephadex G-50 separated the free labeled inhibitor from both lactoperoxidase and free label. The peak fraction (8.9×10^7 cpm) was used for the first experiment. The fractions immediately before and after the peak fraction were pooled and 75 % of this volume (8.6×10^7 cpm) was used for the

second experiment.

Two healthy female volunteers, aged 27 and 29 years, were used. A large bore i.v. catheter was placed in a peripheral arm vein. ^{125}I -PSTI was infused over 30 seconds, and sampling was performed every 10 minutes for 3 hours. Additional samples were taken at 24 and 48 hours. Urine was collected as often as feasible for up to 55 hours. Volumes of each void were recorded and 1 ml aliquots assayed in a γ -counter.

Samples of serum and urine were gel filtrated on a Sephadex G-50 column (0.9 x 60 cm) using a Tris-HCl buffer, pH 7.4. Serum from 10, 30, 45, 60 and 180 minutes and urine from 6 and 18 hours post infusion were gel filtrated. We found that PSTI was rapidly eliminated from the circulation ($T_{1/2}$ 6 min.). Within 30 min. only 30% of the infused counts remained. The total remaining counts represent both whole protein and labeled degradation products as demonstrated by gel filtration (VI). Labeled protein was eliminated within 2 hours. Within 24 hours the total counts had fallen to 2 to 4% of the infused counts. Simultaneous radioactivity rapidly appeared in the urine with 75% of the infused counts present in the urine within 24 hours.

These findings agree with the work of others who described the elimination of canine PSTI and Trasylol (40,96). It might be argued that during acute pancreatitis PSTI is complexed with proteases or protease inhibitor complexes and therefore its elimination is delayed. However, it has been shown that the vast majority of PSTI is displaced from protease complexes when introduced to serum (36,40). Further, Ohlsson has shown that α_2 -M-trypsin complexes are rapidly eliminated by the RES ($T_{1/2}$ 10 min) (75), and we have shown that there is only minimal incorporation of PSTI into such complexes anyway. Also, PSTI within such a complex is probably not available to immunoglobulin detection as α_2 -M bound trypsin cannot be detected using immunologic means. Therefore these findings make PSTI an unlikely candidate as a late diagnostic indicator of acute pancreatitis.

E. Immunoreactive PSTI in the pancreas juice of patient volunteers collected by Endoscopic Retrograde Choledcho-Pancreaticography (ERCP) (VII, VIII)

Protocols for the routine evaluation of pancreatic function based on duodenal fluid analysis are well known (34). However, duodenal juice does not contain free PSTI, as trypsinogen is rapidly activated by enterokinase and complexes the available PSTI. Therefore, in order to study the production of PSTI pure pancreas juice obtained via direct selective cannulation of the duct of Wirsung is required.

Protocols previously described for this type of experimental collection are generally complicated and do not allow for the use of radio-contrast material as its effects on the pancreas have been only minimally studied (86,94). This adds significantly to the time of collection, discomfort to the patient and may interfere with requisite morphologic studies when patients are referred for diagnostic testing.

We wished to develop a protocol which fit the following requirements:

- it should, 1) be simple and easily standardized,
- 2) not interfere with the requisite morphological studies for which the patient was referred,
- 3) not add to the morbidity of the study or discomfort of the patient,
- 4) not interfere with the quantitative analytic methods necessary to evaluate the juice or with the juice itself.

In our initial study fourteen patient volunteers referred for ERCP had normal morphological findings and no history of chronic or current acute illnesses. Six of these patients underwent their studies without the use of a pre-stimulation contrast injection. The remaining 8 underwent collection following a selective pancreatic duct injection of Cerebral 280 radio-contrast media.

Stimulation to aid juice flow and assure good secretory protein content was intravenous secretin and CCK-PZ infused over 1 min. (1 CU/Kg of each to a maximum of 75 CU). Pre-medication was atropine and droperidol supported by diazepam as needed. Collection parameters were established based on previous work from our laboratories

by Eddeland and Wehlin (42). They demonstrated maximum secretion of protein 5 to 7 minutes following secretin and CCK-PZ stimulation. Secretin stimulated juice flow peaked at about the same time. Juice was collected in 3 periods: 0 - 3.5 min, 3.5 - 9.0 min, and 9.0 - 10.0 min post stimulation.

Analysis of the juice for immunoreactive trypsin, PSTI and pancreatic elastase using radialimmunodiffusion demonstrated equivalent concentrations of these proteins in Periods II and III juice specimens, but not in Period I where marked reduction in concentration was found when contrast was used. Period II samples contained the peak protein concentration. Equivalent results were found for both groups in Period II juice collections when radioimmunoassay (RIA) (trypsin, PSTI and elastase) or enzymatic analysis (α -amylase) was used.

Roentgenopographs demonstrated at least some contrast in Period II tubes, but contrast did not affect the stability of the juice during a 30 minute bench top incubation, or for periods up to 1 year in storage at -20°C . Nor did the presence of contrast affect the qualitative or quantitative character of the immunochemical or enzymatic techniques used. The collection protocol added less than 15 minutes to the procedure and did not appreciably add to patient discomfort. Therefore, this protocol met the desired requirements and was used for routine collection of pure human pancreas juice for further study.

84 patient volunteers referred to the ERCP unit of the Roentgen Department of Malmö General Hospital were entered into the protocol. Of these, juice was collected successfully in 71 patients. Ten of these were rejected due to experimental design error, contamination of the juice with bile or incomplete work-up. The 61 remaining patients were classified into one of 5 diagnostic categories as follows:

Normal (no demonstrable diseases)	9
Pancreatic Carcinoma (by histology)	9
Other Carcinoma (by histology) *	8
Hepatobiliary Disease (HBD) *	32
Pancreatitis (3 with ERP changes of chronic disease)	6

* 3 patients included in both categories

Balldin, *et al* have recently described an overall effect of cigarette smoking on the pancreas gland which results in markedly elevated serum trypsin-like immunoreactivity following secretin stimulation (9). These results were recently confirmed by Andriulli, *et al* (5).

Patients were therefore divided into groups based on:

smoking history:

- Smokers (39) - those smoking more than 1 pack-year and up until the time of their examination.
- Non-smokers (18) - those who never smoked or had smoked less than 1 pack-year and had stopped more than 3 years prior to their study.
- Neither (4) - those giving an unreliable history or who could not be clearly entered into one of the above categories.

and age:

- ≥ 65 years of age (20)
- < 65 years of age (41)

All patients received atropine and droperidol, supplemented by diazepam as needed. Several patients received glucagon, however, all received this drug more than 15 to 20 minutes pre-stimulation, so that its inhibitory effect on the pancreas in response to

CCK-PZ was probably minimal and was ignored for the sake of this study (42).

All samples were analyzed in batch using RIA's on serial dilutions of samples for PSTI, trypsin and pancreatic elastase.

α -Amylase was measured using the commercially available Phadebas technique. Analyses were run in duplicate and samples were thawed only one time for analysis.

No differences were apparent between groups based on smoking history or age.

These results indicate that the increase of amylase activity and trypsin-like immunoreactivity in serum after secretin stimulation in smokers (9) is not due to differences in the enzyme concentration in the juice but rather to an increased interstitial leakage and backflow of the proteins after stimulation.

When patient data was analyzed according to diagnostic category there were several significant findings. All patients with pancreatic disease had a trend toward decreased juice concentrations of trypsin, PSTI and pancreatic elastase. α -Amylase did not demonstrate this trend indicating non-parallel secretion between these proteins (VIII).

Trypsin and elastase levels were significantly less in the juice of patients with chronic pancreatitis, pancreatic carcinoma and hepato-biliary disease than in the normal group ($p = .05$). However, no statistical differences were discernible between the diseased groups. These results agree with the work of others who noted significant drops of exocrine protein or "enzyme" output in patients with chronic pancreatitis and pancreatic carcinoma (27,31,32,34,82).

On the other hand the PSTI concentration was remarkably lower in the juice of patients having pancreatic carcinoma than in patients having other pancreatic diseases or hepato-biliary disease

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Whether this decrease in PSTI is secondary to a selective decrease in production, inhibited secretion or consumption of PSTI due to local pancreatic inflammation in the area of the tumor remains to be determined. However, as noted above (IV), we have recently demonstrated that our rabbit anti-human PSTI serum is capable of detecting only free PSTI. We have also demonstrated that aprotinin is capable of rapidly displacing PSTI from trypsin *in vitro*. When aprotinin was placed in samples having low PSTI content, either no or a limited increase in PSTI concentration was noted. Therefore, it seems less likely that PSTI is decreased in tumor patients due to local complexation of the inhibitor, as degeneration of the immunocompetent part of the molecule by trypsin is not a remarkably rapid process. Whether this finding is of diagnostic significance or simply a curiosity remains to be tested. The need for such a study is evident given our current inability to detect pancreatic carcinoma at an early and therefore curable stage.

F. Immunohistologic localization of PSTI and trypsin in the human pancreas (IX)

PSTI is secreted in .1 to .8% of the total trypsin concentration of normal pancreas juice. This is probably a great molar excess compared to the concentration of active trypsin and therefore PSTI is able to perform a protective function. When the juice reaches the duodenum enterokinase rapidly activates trypsinogen to trypsin which then complexes and overwhelms the accompanying PSTI. In order for PSTI to perform a protective role effectively it should be distributed similarly to trypsinogen throughout the pancreas. Therefore, we chose to study the distribution of these two antigens (PSTI and human cationic trypsinogen), using an immunoperoxidase technique as described by Sternberger and Mason (68,92).

Previous authors have successfully used similar methods to study exocrine protein distribution in the pancreas of cattle (62,67), pig (99) and rat (13). Formalin fixed and paraffin embedded human pancreas tissue removed at operation was obtained from the Department of Pathology. These specimens were from patients operated on for either chronic pancreatitis or pancreatic carcinoma. Five micron sections were made, dewaxed and exposed to the appropriate antiserum in dilutions ranging from 1:100 to 1:2000. Controls were handled identically with antigen adsorbed antiserum (1:100). Background was reduced using pepsin digestion as described by Reading (81). Commercial PAP complexes were utilized and developed with 3,3'-diamino-benzidine tetrahydrochloride to give a positive color.

Both PSTI and cathodal trypsin were broadly distributed throughout all acinar tissue. Serial sectioning allowed us to demonstrate that both antigens were present in the same cells. This was anticipated as this distribution facilitates the protective role of PSTI.

Incidental review of some of the sections which were involved with tumor revealed no staining in tumor cells *per se*. This raised another question, i.e. "do pancreatic tumors contain acinar sec-

retory proteins?" This question is important both academically and clinically. The cells of origin of pancreatic carcinoma in many animals and man are in question. While it is generally agreed that most animal tumors are of acinar cell origin, tumors of man and hamster are believed to be of ductal origin (16,17,30,33,71, 79,83). These beliefs are based on light and electron microscopic evidence which demonstrates tubular structures characteristic of ductal epithelium, the absence of zymogen granules and cell height which is decreased from that of acinar cells (16,17,77,78,84).

Bockman (15) has demonstrated that these characteristics, especially the formation of tubular structures, may be normal variants, or at least non-specific, and therefore invalid criteria with which to differentiate acinar versus ductal cell origins. Hansen (57) has recently done immunohistochemical studies of rat acinar cell tumors and demonstrated several secretory enzymes in both normal and tumor cells. Some tumor cells had no demonstrable characteristics of acinar cells, i.e. loss of zymogen granules, but still contained the enzymes. We stained human exocrine ductal and undifferentiated pancreatic tumors for PSTI and trypsinogen using the same immunoperoxidase technique. None of the tumors contained these antigens.

These findings represent a limited experience and much more work needs to be done. However, the consistent lack of staining in the major area of tumor for all 8 specimens is remarkable. Our observations so far support the concept that known human tumors are not of acinar cell origin, i.e. the result of acinar cell de-differentiation, and that the tumors of lower animals are likely of a different origin.

IV. CONCLUDING REMARKS

As noted in the Introduction, Laskowski has hypothesized that protease producing tissues also produce low molecular weight protease inhibitors. The primary function of these inhibitors appears to be local tissue protection. The overall conclusion of this series of works certainly agrees that PSTI serves such a role.

In most mammals the pancreas produces numerous enzymes but only one low molecular weight inhibitor, the trypsin specific inhibitor, PSTI. Pigs and cattle also produce a second low molecular weight inhibitor, aprotinin, which inhibits trypsin, chymotrypsin and leukocyte elastase. We have isolated a PSTI analogue in rat pancreas. It behaves in a similar fashion to human PSTI in normal animals and during experimental pancreatitis.

When taurocholate alone is used to induce pancreatitis, the ability of activated trypsin to overload local PSTI appears to be a part of establishing the disease. Under controlled conditions infusion of buffer alone did not produce severe pancreatitis despite serum elevations of immunoreactive trypsin, PSTI and α -amylase but no release of active proteases. The pancreatitis seems to result from a non-traumatic insult to the gland which begins a chain reaction involving activated proteases. Therefore, taurocholate duct infusion appears to be a good model with which to study pancreatitis. Of course a remaining key question is 'what causes activation of trypsinogen in this model?'

In vitro human PSTI is capable of inhibiting human α_2 -M-bound trypsin to a limited extent. Trypsin bound to α_2 -M in a molar ratio 1:1 is more susceptible to inhibition by PSTI than when it is bound in a molar ratio 2:1. Even this limited inhibition was a slow process needing about one hour to reach equilibrium which argues against a biologically significant role for PSTI as an inhibitor of these complexes. This conclusion is supported by the finding that trypsin was consistently demonstrable in the α_2 -M fraction of the peritoneal exudates from patients with severe acute pancreatitis but not PSTI.

We found exceedingly low levels of PSTI in the pancreas juice of patients having pancreatic carcinoma. These levels were signifi-

cantly lower than those of the other proteins tested and lower than PSTI levels in the other diseases tested. Prospective studies as well as studies on the mechanism of this depression are necessary to determine if any diagnostic potential exists for this finding.

Tissue staining using a specific immunoperoxidase method found PSTI to be broadly distributed throughout the gland, its distribution paralleling that of trypsin. Such a distribution offers an efficient method of inhibiting prematurely activated trypsinogen. However, whether this PSTI is packaged in the zymogen granules on the endoplasmic reticulum or is a cytoplasmic inhibitor has not been determined yet. This is an important area of investigation since if PSTI is a cytoplasmic inhibitor, cellular disruption may lead to decreased peri-zymogen concentrations of inhibitor allowing a local buildup of the enzyme. Such a picture would be a perfect setup for the beginning of acute pancreatitis.

Our investigation on the distribution of PSTI in the gland further revealed that no staining was present in exocrine tumors, whether 'undifferentiated' or 'ductal' in origin. These findings lend support to the belief that human pancreatic adenocarcinoma is of ductal origin.

In the concluding remarks of Eddeland's thesis, which discussed some of the pioneering work on human PSTI, he noted that further clinical studies "may prove rewarding not only in acute pancreatitis but also in chronic inflammation and tumors of the pancreatic gland" (35). We have found this to be the case and believe the major questions deserving investigation concerning PSTI in the adult now relate to two areas: 1) the use of PSTI as a diagnostic tool for pancreatic carcinoma, and 2) the ultrastructural localization of PSTI and how this relates to the pathogenesis of clinical and experimental pancreatitis.

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ISOLATION AND PARTIAL CHARACTERIZATION OF THE PANCREATIC SECRETORY TRYPSIN INHIBITOR IN THE RAT

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Key words: Trypsin inhibitor; Inhibition specificity; Protein secretion; (Rat pancreas)

Isolation in a 55% yield of the low molecular weight pancreatic secretory trypsin inhibitor was achieved by gel filtration of an acid extract of whole inactive rat pancreas juice on Sephadex G-50 at pH 2.5 followed by desalting and ion-exchange chromatography on SP Sephadex C-50 at pH 4.5. Two distinct chromatographic fractions were obtained, labeled fraction 1 and 2. Fractions 1 and 2 showed three, respectively two, distinct closely migrating cationic bands on gel electrophoresis in barbital buffer, pH 8.6. Each fraction demonstrated one band on polyacrylamide disc electrophoresis at pH 4.6. The inhibitor is homogenous on gel filtration and on the basis of its stoichiometry with active site titrated rat anionic trypsin. Its molecular weight is approx. 6024. The amino acid composition is included. Rat pancreatic secretory trypsin inhibitor is trypsin-specific and interacts on a 1:1 molar basis with rat trypsin. It is a good inhibitor of bovine trypsin but a poor inhibitor of human cationic trypsin and its binding to trypsin is reversible by acidification. Like other inhibitors of this sort, it is present in about 0.1–0.2% of the total protein content of the juice, and normally exists in its free form. A simple procedure for the production of antiserum to the inhibitor which is a poor antigen is also described.

Introduction

The low molecular weight trypsin inhibitor of the acid-stable 'Kazal' type has previously been described in several species. These include man, cow, pig, dog and ox [1–7]. Although a similar inhibitor was described in the rat as early as 1958 by Grossman [8], it has never been isolated or characterized in any detail. The inhibitor is a secretory protein of the pancreas which is believed to be produced in the acini of the gland and for this reason it is commonly called pancreatic secretory trypsin inhibitor.

Pancreatic secretory trypsin inhibitor is believed to act as a local inhibitor within the pancreas and to protect against the deleterious effects of prematurely activated trypsin. It has been studied in various diseases of the pancreas, especially pancreatitis, both directly by immunochemical techniques in man [9,10] and indirectly in rat, using enzyme inhibition techniques [11]. However, defining the pathophysiological and biochemical basis of pancreatitis and the role of the inhibitor in this process remains a much debated problem.

The problems associated with the study of pancreatitis are manifold. One major difficulty is that the nature of the disease requires the death of animals upon completion of the experiments. This leads to costly programs in order to furnish enough animals to make statistical analysis meaningful.

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Further, studies in humans are fraught with both ethical and legal problems. We have chosen to develop an economical immunochemical system, using the rat, by which we can study trypsin inhibitor in various disease states of the pancreas. Therefore, the purpose of this study was to isolate and partially characterize the trypsin inhibitor of rat pancreas juice.

Materials and Methods

Sephadex G-25, G-50, G-75 and SP Sephadex C-50 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Benzoyl-DL-arginine-*p*-anilide-HCl (BAPNA) was purchased from FLUKA AB. Freund's complete adjuvant was supplied by DIFCO Labs, Detroit, MI, USA. Bovine trypsin was a product of The Swedish Pharmacy Corporation. Agarose was obtained from the FMC Corp., Rockland, MD, USA. Agarose gel electrophoresis was carried out according to Johansson [12] in a barbital buffer pH 8.6.

Polyacrylamide disc electrophoresis was run at pH 4.6 using an amperage of 5 mA/tube. The running time was 1.5 h, following a 3 h pre-run. Gels were fixed for 1 h in picric acid and stained overnight with 0.2% Coomassie R-250 in 50% methanol.

Densitometric scans of the stained gels were performed using a Zeinh Soft Laser Scanning Densitometer (Biomed Instruments, Chicago, IL, USA).

The amino acid composition was determined according to the procedure of Spackman, et al. [13] using a Jeol 5AH automated amino acid analyzer. Values were calculated on the basis of 24 and 72 h hydrolysates in constantly boiling 6 N HCL. Cystine and methionine residues were determined following performic acid oxidation [14].

Human cationic trypsin was isolated according to Ohlsson and Skude [15].

Rat anionic trypsin was isolated according to Genell [16], with the exception that the first step involved a gel filtration of whole rat pancreas juice. The same antisera against human trypsin inhibitor [6,9], Trasylol [17] and rat trypsin [16] as characterized before were used.

Radial immunodiffusion was performed accord-

ing to Mancini [21] and was used to trace these proteins in gel filtration fractions.

Trypsin inhibition studies were performed according to Erlanger [18] by titrating increasing amounts of the inhibitor against a fixed amount of active site titrated trypsin [19]. The reaction was started by the addition of the substrate BAPNA, stopped after 20 min by the addition of 30% acetic acid and the colorimetric results read at 410 nm in a spectrophotometer.

Chymotrypsin inhibition was determined using a Test-combination kit with instructions according to the company. (Boehringer Mannheim).

Radioactive labeling was performed using a lactoperoxidase technique [20]. Approximately 50 μ g of trypsin inhibitor was labeled using 37 MBq 125 I. Free radionuclide and lactoperoxidase were separated from the labeled protein by gel filtration.

Ultrafiltration was carried out using an Amicon chamber equipped with a UM-2 membrane.

All reactions were carried out at 4°C except the trypsin inhibition studies, which were performed at ambient temperature.

Animals. Female Wistar rats were used weighing 190–220 g.

Rat pancreatic juice was collected on dry ice following proximal ligation and distal cannulation of the hepatopancreatic duct, as previously described by Genell [16]. A PD-10 (0.11 I.D.) polyethylene catheter was used, and brought out through the tail via a specially designed stay tag which allowed the animal to eat and drink ad libitum and to have a free range of motion for almost the entire length of her tail. No animal was kept in the cage for more than 6 post operative days. Each animal secreted approx. 15–30 ml of clear colorless juice containing approx. 10 g protein/l per 24 h, starting 12–24 h post cannulation. The juice was lyophilized and stored at -20°C until used.

Isolation of the inhibitor

Gel filtration. Approx. 500 ml of lyophilized sample was divided into three equal batches. All batches were handled identically. The lyophilized product was dissolved in 25 ml of distilled water. The pH of the solution was then slowly lowered to 2.5 by the addition of 4 M HCl under constant

stirring on a magnetic stirrer. A large precipitate formed and was centrifuged off at 8000 rpm. The precipitate was washed with 10^{-2} M HCl twice and the wash combined with original supernatant. The entire batch was then concentrated to about 20 ml using ultra filtration and again centrifuged to both clear and degass solution. The entire supernatant was then charged directly to a Sephadex G-50 column (5.0×100 cm) previously equilibrated with 10^{-2} M HCl containing 0.5 M NaCl and 0.01 M CaCl_2 (Fig. 1). The flow rate was 36 ml/h and the effluent was collected in 6 ml fractions. All fraction were tested for trypsin inhibition using BAPNA as substrate.

The inhibitory fractions were pooled and stored at -20°C until all three batches had been handled in a similar fashion.

Ion exchange. The inhibitory fractions were pooled, concentrated by ultrafiltration and the buffer exchanged on a Sephadex G-25 desalting column (2.6×40 cm) which had been equilibrated with sodium acetate buffer, pH 4.5, 0.05 M containing 0.01 M CaCl_2 (Fig. 2). The concentrate was charged to a SP Sephadex C-50 column (2.6×40 cm) which had been equilibrated with the same buffer. Following elution with one column volume of the starting buffer, a NaCl gradient was begun (0.0–0.6 M), using a total volume of 600 ml. All fractions were again tested for trypsin inhibitory capacity and the active fractions were pooled. Inhibitory fractions were found in two distinct

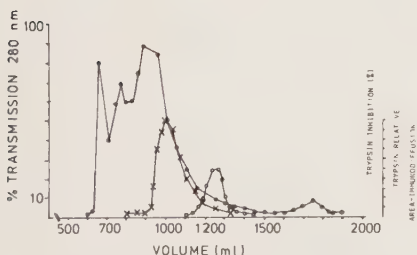


Fig. 1. Elution profile of rat pancreas juice subjected to Sephadex G-50 gel filtration at pH 2.5 (5.0×100 cm) in HCl 10^{-2} M containing 0.01 M CaCl_2 and 0.5 M NaCl. Ultraviolet profile (●—●). Elution volume of trypsin inhibitory fractions (○—○) BAPNA as substrate. Trypsin containing fractions determined by radial immunodiffusion (×—×).

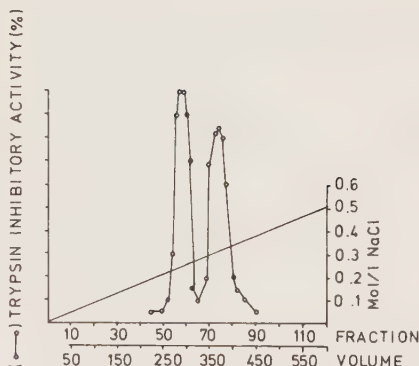


Fig. 2. Elution of trypsin inhibitory pool of fig. 1 from ion exchange column (2.5×40 cm). SP Sephadex C-50 sodium acetate buffer, pH 4.5, containing 0.01 M CaCl_2 as starting buffer. Following 1 column volume a NaCl gradient was begun with 300 ml of starting buffer to mix with 300 ml of starting buffer containing 0.6 M NaCl.

peaks and were labeled fraction 1 and fraction 2, respectively. These peaks were pooled separately, desalted and lyophilized.

Production of antiserum. 0.5 mg of the pancreatic secretory trypsin inhibitor isolate fraction 1 was dissolved in a Tris-HCL buffer, pH 7.6, containing 0.5 M NaCl and charged to a Sepharose-bovine trypsin affinity column. Following several column volumes of elution with the starting buffer, the inhibitor was eluted in a single peak from the column using 0.5 M formic acid containing 0.5 M NaCl and 0.1 M CaCl_2 . The eluate was concentrated, desalted and lyophilized. The product was then dissolved in 500 μl of distilled water and emulsified with an equal volume of Freund's adjuvant. The entire preparation was then injected subcutaneously into the back of rabbit. A booster was given at 4 weeks and harvesting of the antiserum was begun 8 weeks after the initial inoculation.

Results

Trypsin inhibitor fraction 1 behaved as three closely migrating cationic bands on agarose gel electrophoresis at pH 8.6. in a barbital buffer.

Fraction 2 demonstrated two closely migrating cationic bands which migrated slightly further in the same system.

Polyacrylamide disc electrophoresis at pH 4.6 resulted in a single well-defined band for each isolate. Fraction 2 migrated more cathodally than did fraction 1 (Fig. 3).

Molecular weight studies. Trace amounts of labeled inhibitor were added to mixtures of Trasylol (M_r approx. 6500) and human trypsin inhibitor (M_r approx. 6200), and the products gel filtrated on Sephadex G-75 (0.9×55 cm). All three components were eluted in the same elution volume. In a second experiment, 100 μ g quantities of the same three unlabeled inhibitors were charged to the same column and the fractions tested using Mancini radial immunodiffusion and the appropriate antiserum. Again, all three components were eluted in the identical elution volume.

Amino acid composition. Due to the small sample availability only fraction 1 was determined.

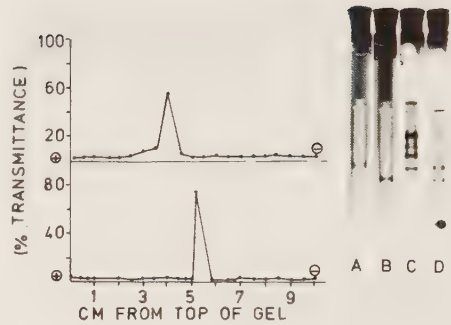


Fig. 3. Polyacrylamide disc electrophoresis of rat trypsin inhibitor (A) fraction 1 (B) fraction 2, (C) whole rat pancreas juice and (D) a mixture of fractions 1 and 2 (pH 4.6). Time of running, 1.5 h. Densitometric scans of fractions 1 and 2 are included.

TABLE I

AMINO ACID ANALYSIS OF TRYPSIN INHIBITOR IN VARIOUS SPECIES AND APROTININ

Amino acids	Rat trypsin inhibitor (Present study)	Human trypsin inhibitor (Ref. 25)	Bovine trypsin inhibitor (Ref. 26)	Porcine trypsin inhibitor (Ref. 4)	Aprotinin (Ref. 27)
Asp	5	8	7	4	5
Thr	4 ^c	4	4	6	3
Ser	4 ^c	3	2	6	1
Glu	5	6	7	7	3
Gly	6	5	5	4	6
Ala	1	1	1	1	6
Val	2 ^b	2	4	4	1
Ile	4 ^b	3	3	3	2
Leu	1	4	4	2	2
Tyr	2	3	2	2	4
Phe	2	1	0	2	4
His	1	0	0	0	0
Lys	4	4	4	4	4
Arg	3	3	3	4	6
Pro	5	3	3	5	6
Cystic acid	6 ^a	6	6	6	6
Met	0 ^a	0	1	0	1
Total	56	56	56	56	58
M_r	6024	6242	6155	6024	6315

^a Determined after performic acid oxidation.

^b 72-h hydrolysis.

^c Extrapolated to zero

The results of this analysis and similar molecules from other species are given in the table.

Inhibition studies. Rat trypsin inhibitor was capable of inhibiting rat anionic trypsin on a mole for mole basis, and could completely inhibit its activity. When tested against rat cationic chymotrypsin, no inhibition of the activity was noted. The inhibitor was a good inhibitor of bovine trypsin, capable of inhibiting 85% of its activity when present in greater than 4-fold molar excess. However, it proved to be a poor inhibitor of human cationic trypsin, being capable of inhibiting only about 30% of its activity in 2.5-fold molar excess (Fig. 4).

Immunochemical studies. Immunelectrophoresis of whole rat pancreas juice versus rabbit anti-rat trypsin inhibitor produced a long bowed precipitate. When fractions 1 and 2 were tested together against the antiserum, a continual precipitate, without spurring, appeared. When fractions 1 and 2 were run separately, each gave a single bowed precipitate (Fig. 5). Ouchterlony double immunodiffusion of fractions 1 and 2 and whole rat pancreas juice resulted in a reaction identity.

Quantitation in pancreas juice using radial immunodiffusion on agarose plates containing anti-rat trypsin inhibitor and 3% poly(ethyleneglycol) demonstrated trypsin inhibitor to be present in a

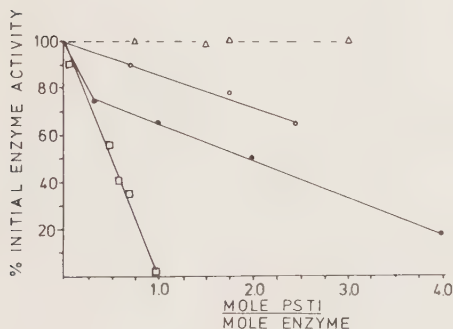


Fig. 4. Inhibition of trypsin by rat trypsin inhibitor (PSTI). A constant amount of trypsin was titrated against increasing amounts of rat trypsin inhibitor. Rat anionic trypsin ($\square$ — $\square$); bovine trypsin ($\bullet$ — $\bullet$); human cationic trypsin ($\circ$ — $\circ$). The inability of rat trypsin inhibitor to inhibit rat chymotrypsin is also demonstrated ($\triangle$ — $\triangle$).



Fig. 5. Immunelectrophoresis of rat trypsin inhibitor fractions 1 and 2 and whole rat pancreas juice in agarose with antiserum against fraction 1. Identical results were obtained with antiserum against fraction 2. (A, fraction 2; B, whole rat pancreas juice; C, fraction 1).

concentration of about 10–20 mg/l. Samples were obtained from healthy animals being fed normal diets ad libitum. Similar testing using urine and serum failed to demonstrate the presence of the inhibitor in these fluids.

Discussion

The isolation of trypsin inhibitor has previously been accomplished in several species [2–4,6]. However, although its existence has been reported in rat [8], it has not, to our knowledge, been isolated or characterized to date. The need to develop an economical model with which to study the role of this inhibitor in diseases of the pancreas led us to pursue its isolation and partial characterization in the rat.

Rat trypsin inhibitor exists in multiple electrophoretic forms which appear to be immunologically identical, as is the case in other species [2,6,7]. This low molecular weight inhibitor (M_r

6200) fulfills the criteria describing trypsin inhibitor (Kazal) type inhibitors, namely, it is an acid-stable, trypsin-specific inhibitor whose binding to trypsin is reversible by acidification. Rat trypsin inhibitor is cationic in a pH 8.6 barbital buffer system on electrophoresis while others are described as anionic in similar systems.

On gel filtration it proved to have a molecular weight in agreement with its amino acid analysis and with similar molecules isolated in other species. Of note is the existence in this inhibitor of histidine, which has been lacking in other species.

Following purification of this molecule we were able to stimulate antiserum production to it only after exposing it to bovine trypsin for a brief period on a trypsin-Sepharose affinity column. Other authors have also described this type of molecule as being a poor antigen. Greene and Fink [22] were able to enhance the antigenicity of their bovine trypsin inhibitor by coupling it to albumin. In this laboratory we have had success using brief trypsin exposure with both the human and now rat trypsin inhibitor (unpublished data). Exposure of trypsin inhibitor to trypsin is known to begin a series of internal hydrolyses which result in the eventual degradation of the inhibitor. This phenomenon results in a 'temporary inhibition' a term coined by Laskowski et al. [23]. It seems likely that this brief exposure to trypsin results in a partial opening up of the molecule, which is known from other work, to be tightly coiled on itself [24], thus exposing its immunogenic site(s).

Our antiserum was specific for rat trypsin inhibitor as judged by immunoelectrophoretic comparison of the isolated forms of the molecule and whole inactive rat pancreas juice. Further, the two major fractions of the inhibitor, as collected from ion-exchange chromatography, gave the reaction of identity on double diffusion.

Using our antiserum for radial immunodiffusion, we were able to quantitate the molecule in whole pancreas juice and to observe its behavior on gel filtration. The inhibitor was eluted in a single symmetrical peak from inactive pancreas juice, and thus appears to exist in its free form in nature. It was present in a concentration equal to approx. 0.1–0.2% of the total protein content. This is similar to the ratios defined in other animals [2,4,6,7].

Using this same technique, attempts to quantitate trypsin inhibitor in other body fluids (urine and serum) failed. This is likely due to the relative insensitivity of the radial immunodiffusion method (1.5–2.0 mg/l) and demands the production of a more sensitive procedure. Efforts are currently under way in this laboratory to develop a radioimmunoassay which will facilitate this problem.

Rat trypsin inhibitor proved to be a specific trypsin inhibitor capable of inhibiting rat anionic trypsin on a mole/mole basis. This emphasizes its role in the normal gland as that of a guardian against premature activation of trypsin.

Inhibition studies against other trypsinases showed that it is a good inhibitor of bovine trypsin, but a relatively poor inhibitor of human cationic trypsin. These species binding differences may play an important role in experimental design concerning the therapeutic use of low molecular weight inhibitors in the face of active trypsin i.e., experimental or clinical pancreatitis.

In summary, the isolation of rat trypsin inhibitor has been accomplished and a monospecific antiserum developed to it. Rat trypsin inhibitor appears to fit the definition of other 'Kazal' type inhibitors. The development of more sensitive methods of detection should allow the study of this molecule in economical experimental models for diseases of the pancreas.

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A Radioimmunoassay for Rat Pancreatic Secretory Trypsin Inhibitor

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Marks WH, Ohlsson K. A radioimmunoassay for rat pancreatic secretory trypsin inhibitor. *Scand J Gastroenterol* 1983, 18, in press.

A radioimmunoassay for the detection of pancreatic secretory trypsin inhibitor (PSTI) in the rat is described. The sensitivity of the assay enables the specific measurement of this inhibitor in the serum of normal rats. The average base-line value for multiple animal serum specimens taken from fasting female Wistar rats being fed conventional diets was $11.6 \pm 6.2 \mu\text{g/l}$. The inhibitor existed in its free form in serum, and PSTI immunoreactivity increased significantly within 2 h of the induction of experimental pancreatitis. The present assay will facilitate the study of PSTI in experimental diseases of the pancreas.

Key words: Pancreatitis; PSTI; RIA; trypsin inhibitor

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Biochemical investigations of the pancreas in both normal and diseased states are greatly aided by the sensitivity and specificity of immunochemical techniques.

Pancreatic secretory trypsin inhibitor (PSTI) is a specific inhibitor of trypsin which is secreted by the acini of the pancreas (1). Its function in the normal gland appears to be that of a safety net, protecting locally against the potentially catastrophic effects of prematurely activated trypsin (2, 3). Several investigations have been performed to help elucidate the role of PSTI in the diseased gland in various species, including man. Most attention has centered on pancreatitis (4–6), although its involvement in other diseases is also of interest, both functionally and diagnostically (7–9). We have described the isolation and partial characterization of PSTI in the rat and also the production of antiserum to it in a previous paper (10). Techniques to study this protein involving radial immunodiffusion or enzymatic inhibition are only sensitive enough to enable study of the pancreatic juice or extracts of the gland itself.

Radioimmunoassay (RIA) for PSTI has been described by Fink for cattle (11) and for man by Eddeland & Ohlsson (4) and later by Kitahara et al. (12). Experimental studies in humans create several ethical problems, whereas the use of large lower animals in numbers sufficient to satisfy statistical requirements can be financially prohibitive.

The purpose of this work was to develop a RIA capable of measuring PSTI in the serum of normal rats and thus to facilitate the study of this inhibitor during experimental diseases of the pancreas.

MATERIALS AND METHODS

Special material. Sephadex G-50 and G-75 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine serum albumin fraction was a product of Miles Laboratories, England.

Biological specimens. Blood samples were obtained from 10 healthy female Wistar rats weighing 180–200 g and 10 healthy male Wistar rats weighing 190–200 g. The animals were on a standard diet and were fasted overnight before

sampling. Light ether anesthesia was induced, and samples were taken from direct cardiac puncture. The samples were placed on ice, and then the serum was separated and stored at -20°C until tested.

Pancreatitis. Female Wistar rats weighing approximately 200 g were used. Anesthesia was induced with chloral hydrate intraperitoneally. A carotid arterial line was placed, brought out through the neck, and connected to a pulsatile infusion pump. A base-line blood sample was obtained, and then taurocholate pancreatitis was induced as described by Aho et al. (13). Sampling of blood (500 μl) via the arterial line was done 15 min and 1 h after induction.

Rat PSTI. Rat PSTI was isolated as previously reported (10).

Rabbit anti-rat PSTI. Rabbit anti-rat PSTI were produced by inoculating rabbits subcutaneously with rat PSTI briefly exposed to trypsin on a trypsin-Sepharose column, as previously described (10). The medium was complete Freund's adjuvant. A booster was given at week 4, and harvesting commenced 8 weeks after inoculation.

Radioactive labeling. Approximately 8 μg of rat PSTI was labeled by using 1 MBq of ^{125}I and a lactoperoxidase method (14) yielding approximately 1 mol ^{125}I to 1 mol protein. Unbound radionuclide was separated from the reaction product by gel filtration on a Sephadex G-50 column (0.9×30 cm) that had previously been exposed to a wash of 2% bovine serum albumin (BSA). Fractions (1 ml) were collected in tubes into which 1 ml of RIA buffer (Tris-HCl, pH 7.4, containing 0.12 M NaCl and 2% BSA) had previously been placed. Aliquots (10 μl) of each fraction were screened, using a well-type scintillation counter. Approximately 70% of the total counts were present in the inhibitor peak. The fraction that was eluted immediately after the fraction of peak activity was divided into small aliquots and used for the assay. This fraction was used to help ensure complete separation of the labeled product from lactoperoxidase. All dilutions were performed using the RIA buffer.

Antiserum titer. The tracer was diluted to yield about 20,000 cpm/100 μl . 100 μl were incubated

together with 300 μl RIA buffer and 100 μl of a 1:2 dilution series of the rabbit anti-rat Kazal from 1:100 to 1:10⁶. Incubation took place overnight at 4°C . Normal rabbit serum (100 μl) diluted 1:200 and sheep anti-rabbit serum (100 μl) diluted 1:5 were added to each tube, and these were incubated overnight. Finally, 500 μl of RIA buffer was added and the reaction mixtures centrifuged at 2500 g for 15 min at 4°C . The supernatant was decanted off and the bound radioactivity counted in a well-type scintillation counter. The titer was 1:10,000, and 90% of the activity could be precipitated at a dilution of 1:1600.

Activity of the antiserum. Several antiserum dilutions were tested against a standard dilution series of PSTI from 25 to 0.75 $\mu\text{g/l}$. A reaction mixture containing 300 μl of diluted antiserum and 100 μl of the tracer, diluted as above, was incubated overnight at 4°C . The double antibody procedure was again carried out as described and the bound activity counted. A dilution of 1:5000, which gave about 60% precipitation of the tracer when no PSTI was present in the test sample, was chosen for the assay.

PSTI in normal rat serum. 100 μl of a 4:1 dilution of serum were placed in a small test tube together with 100 μl of the labeled tracer, diluted to 20,000 cpm/100 μl , and with 300 μl of the antiserum in a 1:5000 dilution. Incubation was allowed to take place overnight at 4°C . On day 2 the double antibody technique was performed as described above. The bound activity was measured. All samples were run in duplicate, and fresh aliquots of the standard dilution series of the antigen were utilized with each assay.

Gel filtration of serum. Fresh serum was obtained by direct cardiac puncture. Approximately 250 μl was loaded on a Sephadex G-75 column (0.9×55 cm). All fractions were 700 μl , and the flow rate was 8 ml/h. The fractions were screened for inhibitor by the RIA. 100- μl aliquots were used, and all were run in duplicate. A small amount of the tracer PSTI was then loaded on the same column and eluted in a similar manner. All fractions were then submitted for gamma counting. (The eluting buffer was in all cases Tris-HCl, pH 7.4 (0.01 M), containing 0.12 M NaCl and 0.01 M CaCl_2 .) Serum samples from

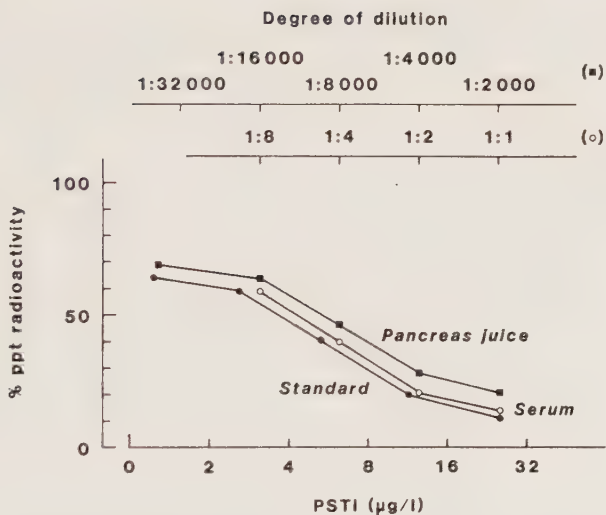


Fig. 1. Dilution curves for immunoreactive PSTI in a standard dilution series (●), serum (○), and pancreas juice in the rat (■). Serum was a pool of serum from three different rats.

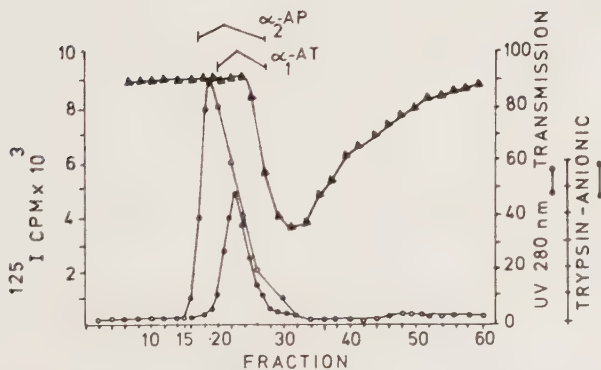


Fig. 2. Rat PSTI ($\blacktriangle$) as determined on serial fractions after gel filtration on Sephadex G-75 (0.9×60 cm) of normal rat serum (250 μl). Elution volumes with peak fractions are indicated by bars for $\alpha_2\text{-AP}$ and $\alpha_1\text{-AT}$, as determined by radial immunodiffusion. The elution pattern of anionic trypsin ($\bullet$) and the ultraviolet profile of serum ($\circ$) are also included for reference.

the pancreatitis group, obtained 15 min and 1 h after induction, were also gel filtered as above, and samples quantitated by the RIA.

RESULTS

Specificity. Dilution curves of rat serum and rat pancreatic juice were essentially parallel to that of a standard dilution series of the inhibitor (Fig. 1). Further, more than 90% of the labeled inhibitor could be precipitated with the antiserum.

Normal levels. Serum levels from 13 female Wistar rats averaged $11.6 \mu\text{g/l}$ (± 6.2).

Gel filtration. Gel filtration of normal serum inhibitor resulted in the immunoreactivity being eluted as a single peak corresponding to the elution volume of the standard pure inhibitor (Fig. 2). Further, this was the same volume as that of the labeled PSTI tracer and followed the volume of anionic trypsinogen.

Levels during pancreatitis. The average circulating level of PSTI in animals with taurocholate pancreatitis 1 h after induction was $50.8 \pm 8.4 \mu\text{g/l}$.

When samples taken 15 min after induction of pancreatitis were gel filtered, a small peak of immunoreactive PSTI was present at the elution volume of free PSTI, the major portion coming later but before the column volume. When 2-h specimens were gel filtered on the same column, all the immunoreactivity was eluted in a volume coming between free PSTI and the column volume (Fig. 3).

DISCUSSION

Both inflammatory and neoplastic diseases of the pancreas are prevalent in Western society and controversial as to their pathophysiological basis. There is a need to develop economic and sensitive models by which to study disease processes in this organ. Past models for study of pancreas diseases have been too insensitive to follow the serum levels of pancreatic secretory proteins (8, 15, 16). We believe that defining the role of the endogenous and specific trypsin inhibitor of mammalian pancreas, PSTI, will prove useful in the understanding of pathophysiological mechanisms in the

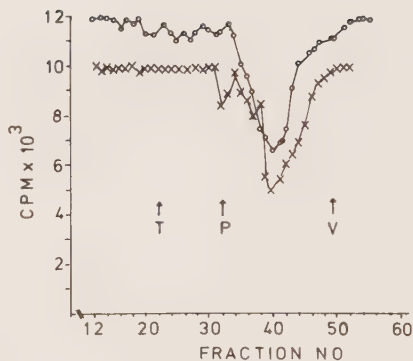


Fig. 3. Gel filtration of serum taken 15 min (x) and 2 h (o) after induction of taurocholate pancreatitis. The column and methods are the same as described for Fig. 1.

pancreas. Further, it is important to define species differences at a functional level to determine better what constitutes a valid disease model, as applies to man. Jalovaara (8) has indirectly evaluated this inhibitor in the rat by studying the trypsin inhibiting capacities of the gland or juice. Others have attempted to analyze it more directly in health and disease by using immunochemical means in man and the dog (4, 11, 12). These studies have either proved too insensitive for study of PSTI in body fluids other than pancreas juice or very costly and/or fraught with ethical-legal problems. In this report we have described a RIA for rat PSTI. We were able to produce antiserum, as previously described (10), to this molecule after a brief trypsin exposure. This technique enables quantitation of the protein in serum and is sensitive, as designed, to $0.75 \mu\text{g/l}$. Dilution curves in serum, juice, and standard pure inhibitor were essentially parallel, indicating minimal cross-reactivity or other interfering substances in the fluids tested with our antiserum. Further, our antiserum was capable of precipitating over 90% of the pure labeled inhibitor.

Gel filtration of fresh serum samples yielded immunoreactive PSTI in a single peak. The elution volume was the same as that of the native free inhibitor, indicating that the protein normally

exists in its free form in the circulation. This is a parallel situation to that of the human (4). However, although there is a marked increase in immunoreactive PSTI in taurocholate-induced pancreatitis, most of the activity was eluted after the free peak, indicating cleavage products of the molecule. This most likely occurs as a consequence of trypsin exposure and resulting internal hydrolysis. Modification of the inhibitor after brief trypsin exposure has been observed in other species in vitro and has been implicated as the mechanism resulting in 'temporary inhibition' of trypsin by PSTI (17). As stated above, we believe we have exploited this reaction in manufacturing PSTI with enhanced immunogenicity. In summary, an RIA has been developed for rat PSTI. This assay should aid in experimental studies of the behavior of this protein in diseased states of the pancreas. Further, it should help define species differences and/or similarities to better apply available experimental models of diseases of the pancreas to man.

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PANCREATIC SECRETORY PROTEINS IN SERA FROM RATS BEFORE AND AFTER INDUCTION OF EXPERIMENTAL PANCREATITIS

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This study has traced the behavior of rat anionic trypsin-like immunoreactivity and PSTI immunoreactivity in the serum of rats undergoing bile-pancreatic duct infusions of buffered solutions with and without the addition of the bile salt, taurocholate. Enzymatic analysis of α -amylase was also observed. A mild pancreatic inflammation resulted following infusion of buffer alone, as determined by gross inspection of the pancreas and the behavior of serum levels of the above proteins. Animals infused with buffer and 4 % taurocholate had major inflammatory changes, including gross hemorrhage into the gland, and marked elevations in serum levels of the three proteins studied. Graphic analysis of the serum levels revealed distinct sharp rises in the serum levels of all three proteins in the taurocholate group. In the buffered saline group only an initial sharp rise was present, followed by a prolonged fall back towards baseline values.

The immunoreactive trypsin in the taurocholate group was present in three fractions with different molecular weights: trypsin in complex with protease inhibitors, trypsinogen and degradation products. PSTI immunoreactivity showed the molecular size of free inhibitor and that of degradation products. The presence of trypsin in complex with protease inhibitors indicates the formation of active trypsin during acute pancreatitis, which is further supported by the presence of degradation products of trypsin and PSTI.

Key-words: Amylase; pancreatitis; protease inhibitors; radioimmunoassay; trypsin; trypsin inhibitors

Numerous models have been developed with which to study acute pancreatitis. A popular model involves the injection of the bile salt taurocholate into the pancreatic duct system of the rat (1). This model provides a simple, economic and reproducible means of studying acute pancreatitis.

Recently, Aho and Nevalainen studied electron microscopic changes in the rat pancreas during taurocholate induced pancreatitis (2). They found widespread progressive necrotic lesions of acinar, ductal and vascular cells, and concluded that the initial lesions were caused by the detergent action of taurocholate on cell membranes. They suggested that later necrotic lesions developed as the result of the action of digestive enzymes.

Satake *et al.* (3) investigated the behavior of rat pancreatic elastase in serum using a similar model. However, in their experiments they utilized active trypsin to induce the pancreatitis, as described by Evander *et al.* (4). The use of active trypsin in this model artificially recruits active proteases into the disease process and also interferes with one of the natural defense mechanisms of the pancreas by complexing the intrinsic trypsin specific inhibitor, pancreatic secretory trypsin inhibitor (PSTI).

The taurocholate model appears to be an excellent one with which to study acute pancreatitis. However, the role of the exocrine pancreatic proteins in its pathogenesis remains undetermined. We believe that their behavior must be explored in order to better determine the application of this model to the study of acute pancreatitis in man.

We have previously described a specific radioimmunoassay (RIA) for PSTI in the rat (5). In this report we describe an RIA specific for rat anionic trypsin. The purpose of this work was to further elucidate this model of pancreatitis by analyzing the behavior in serum of the pancreatic proteins, trypsin, PSTI and α -amylase using only taurocholate as the initiator of the disease.

MATERIALS AND METHODS

Rat-anionic trypsin was isolated according to Genell *et al* (6). A standard dilution of the DFP inactivated enzyme was made from 100 to 1.5 $\mu\text{g/l}$ and aliquots stored in 10^{-3}HCl containing 0.02 M CaCl_2 at -20°C .

^{125}I labelling. $\sim 7\ \mu\text{g}$ of DFP-inactivated rat-anionic trypsin was labelled with 1 mCi of ^{125}I using a lactoperoxidase method (7). Gel filtration on Sephadex G-50 (0.5 x 30 cm) was used to separate unbound ^{125}I . The peak count fraction was stored in small aliquots and used as tracer. Both the labelled enzyme and DFP inactivated enzyme were relatively unstable, demanding fresh preparation to be made monthly.

Antiserum. Rabbit anti-anionic trypsin was prepared according to Genell (6). The second antibody precipitation reagent, sheep anti-rabbit immunoglobulin, was available at the laboratory.

Avidity of antiserum. A 1:2 dilution series gave a titer of 1:200,000. Approximately 90% of the label was precipitated at a dilution of 1:25,600, and 60% at 1:100,000. The latter dilution was chosen for the assay. The K_a was calculated using a Scatchard plot as approximately $2.7 \times 10^{10}\ \text{l/M}$.

Radioimmunoassay procedure. All dilutions were made with a 0.01 M Tris/HCl buffer, containing 0.12 M NaCl, 0.005 M EDTA, 150 mg Trasyol/l and 2 g bovine serum albumin/l at pH 7.4. The labelled DFP-inactivated trypsin was used as tracer in a dilution giving around 200,000 CPM/ml. The antiserum was used in a dilution of 1:100,000 precipitating about 60% of the radioactivity in the assay, when no additional unlabelled trypsin was present. Unlabelled DFP-inactivated trypsin in different dilutions was used as standard. Antiserum dilution 200 μl , tracer dilution 100 μl , 100 μl standard or sample and 100 μl of normal rabbit serum diluted 1:250 were incubated at $+4^\circ\text{C}$ for 24 hrs and then 100 μl of sheep antirabbit immunoglobulin was added. After another 24 hrs of incubation at $+4^\circ\text{C}$, 500 μl of the dilution or buffer was added and the samples were centrifuged for 15 min at $2500 \times g$. The supernatant was decanted and the radioactivity of the precipitate was measured.

Sensitivity of antiserum. Using a dilution of tracer such that 100 μ l yielded 20,000 cpm/100 μ l, a linear plot for a standard dilution series could be achieved over a range from approximately 6 μ g/l to 100 μ g/l.

α -Amylase. The Phadebas test kit produced by Pharmacia Fine Chemicals (Uppsala, Sweden) was used. Serum sample size was 10 μ l and the test was run according to the instructions of the company with the exception that incubation was carried out at 37.°C in a water bath for only 10 min.

Serum amylase. The assays were established so specimens could be evaluated in at least 1:10 dilutions. This allowed for small sample size and facilitated multiple samplings without endangering the animals.

Normal values. Ten female and 10 Wistar rats were used to establish baseline values. Following light ether anesthesia, blood was drawn by direct cardiac puncture. The serum was separated and analyzed for trypsin, PSTI and α -amylase.

Experimental animals. Following pilot experiments to establish the method, 15 Wistar rats of both sexes weighing 190 to 220 g, were used. Ten were given taurocholate in their pancreatico-bile duct system injection as described below. Five animals were handled identically but without taurocholate and will be referred to as "controls".

Pancreatitis. The protocol was as described by Aho and Nevalainen (2) and Evander and Ihse (4) with minor modifications. Following an overnight fast during which animals had free access to water, anesthesia was induced using intraperitoneal Mebumal®. Prior to opening the abdomen a p-50 polyethylene catheter was placed in a carotid artery and baseline blood sample obtained. The abdomen was then entered through a short upper mid-line incision and the duodenum and common bile-pancreatic duct identified. The duct was obstructed proximally using a vascular clip and a p-50 polyethylene catheter introduced transduodenally into the distal duct. Four hundred microliters of a 4 % taurocholate buffer solution were instilled at 5.3 μ l/sec. for 75 sec. using an automatic pump. The animal was secured in a specially built cage, and blood samples

obtained at 2, 15, 30, 60, 120 and 360 min. post duct infusion. Sample size was 300 μ l of whole blood for each of the 7 samples. Serum was removed immediately, an aliquot removed for α -amylase measurement and the rest of the sample frozen immediately at -20°C .

Gel filtration. Serum samples were gel filtrated using G-75 Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) and a 0.9×60 cm column. Eluting buffer was Tris-HCl, pH 7.4, 0.01 M containing 10^{-2} M CaCl_2 0.5 M NaCl. The rate of elution was 8 ml/hr. Fraction volume was 0.7 ml. All fractions were analyzed in duplicate. Student's t test was used for statistical analysis.

RESULTS

Assays. The fasting value of anionic trypsin immunoreactivity for the 20 normal Wistar rats was 126 (± 18) $\mu\text{g/l}$. Dilution curves of rat pancreas juice, serum and standard DFP inactivated trypsin were essentially parallel indicating that the substance being measured was the same in each case.

The minimum detectable amount of anionic trypsin immunoreactivity was 6.0 $\mu\text{g/l}$ or 20-fold less than the normal value.

Values for PSTI and α -amylase in this same group were 15 (± 4) $\mu\text{g/l}$ and 5200 (± 500) units, respectively.

Gross findings. All animals were inspected grossly at the completion of the experiment. In the taurocholate group each had several milliliters of sero-sanguinous fluid in the abdomen. The gland was grossly edematous with multiple patchy areas of necrosis. Diffuse "fatty necrosis" was also evident. Animals without taurocholate had slightly edematous glands and the peritoneal cavity was free of excess fluid.

Behavior of proteins:

Anionic trypsin. Baseline levels of anionic trypsin were 149.5 (± 7.2) $\mu\text{g/l}$ for the taurocholate group. Following infusion of the taurocholate solution, values rose sharply over the first 15 min. to 382 (± 26.1) $\mu\text{g/l}$. Values then continued to elevate to a maximum of 3330 (± 260.6) $\mu\text{g/l}$ by the end of 6 hours. Control animals likewise had a small sharp rise on infusion of saline. Values rose from a baseline of 208 (± 23.4) to 373 (± 47.4) $\mu\text{g/l}$ at 15 min. By 60 min. a significant difference was apparent between the taurocholate and control groups with values of 1825 (± 261.5) $\mu\text{g/l}$ and 451 (± 97.6) $\mu\text{g/l}$, respectively ($p < .05$). At 30 min. a maximum rise to 612 (± 162.2) $\mu\text{g/l}$ was reached by the controls, with their values falling slowly to 257 (± 38.7) $\mu\text{g/l}$ at 6 hours (Fig. 1).

Pancreatic Secretory Trypsin Inhibitor (PSTI). The baseline level for the taurocholate group was 18.8 (± 3.2) $\mu\text{g/l}$. Values then rose sharply from 23.8 (± 3.3) $\mu\text{g/l}$ at 2 min. and 38.0 (± 3.6) $\mu\text{g/l}$ at 30 min. At 6 hours the value was 68.4 (± 8.6) $\mu\text{g/l}$. On the contrary, the buffer group demonstrated a small rise from

their baseline value of $23.2 (\pm 3.1) \mu\text{g/l}$ to $26.2 (\pm 3.1) \mu\text{g/l}$ at 2 min. A maximum rise was noted at 1 hour of $33.5 (\pm 3.6) \mu\text{g/l}$. Values then fell to baseline levels within 6 hours (Fig. 2). Values were significantly different at 60 min. ($p < .02$).

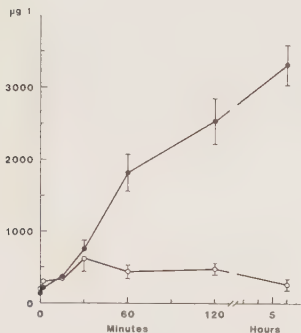


Fig. 1. Serum anionic trypsin immunoreactivity in rats receiving biliary-pancreatic duct infusion of buffer alone (o - o), and buffer with 4 % taurocholate (● - ●). Values are expressed as $\mu\text{g/l}$ and were determined using RIA.

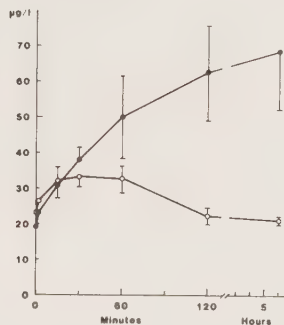


Fig. 2. Serum PSTI immunoreactivity in rats treated as in Fig. 1. Values are expressed as $\mu\text{g/l}$ and were determined using RIA.

α -Amylase. Baseline serum amylase was $4971 (\pm 337)$ units/l for the taurocholate group and $4600 (\pm 285)$ units/l for controls. There was virtually no change between the baseline and two minute values in either group. By 15 min. the control group had elevated values to $4925 (\pm 193)$ units/l, and the taurocholate group was $5343 (\pm 252)$ units/l. At 30 min. the control and taurocholate values began a significant divergence ($p < .02$) with the control values reaching a maximum of $5600 (\pm 305)$ units/l between 1 and 2 hours, and then falling to $5100 (\pm 305)$ units/l at 6 hours post infusion. The taurocholate group, however, maintained a rapid increase between 30 min. and 6 hours with maximum values in excess of 18,500 units/l at 6 hours (Fig. 3).

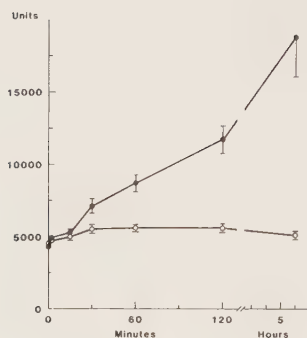


Fig. 3. Serum α -amylase activity in rats treated as in Fig. 1. Values are expressed as U/l and were determined using a commercial enzymatic technique (see Materials and Methods).

Gel filtration. Gel filtration of aliquots of fresh normal serum resulted in the elution of a solitary peak for immunoreactive anionic trypsin (Fig. 4). Serum from animals with pancreatitis demonstrated a massive increase in trypsin immunoreactivity. Some of this activity was eluted earlier than the elution volume of free trypsin, and the majority being eluted as a large broad band beginning at the elution volume of trypsin and ending slightly before the column volume (Fig. 4).

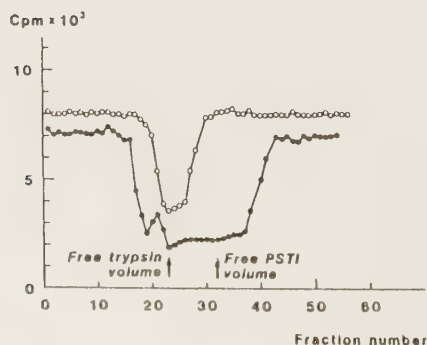


Fig. 4. Anionic trypsin immunoreactivity in fractions from gel filtration of rat serum, normal (200 μ l) (o - o), and 2 hours after the infusion of buffer containing taurocholate, (200 μ l diluted 2:1) (● - ●). (For details; see Methods).

PSTI immunoreactivity eluted as a solitary peak coming after that of trypsin and before that of the column volume. A marked increase in PSTI immunoreactivity was found with induction of pancreatitis. At 15 min. a small amount was present in the free PSTI volume with the majority coming later and running almost to the column volume. By 2 hours the absolute values of PSTI immunoreactivity had increased, but the total amount was eluted in a broad peak, again eluting after the PSTI elution volume but before the column volume (Fig. 5).

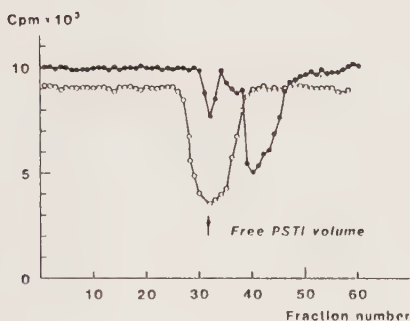


Fig. 5. PSTI immunoreactivity in fractions from gel filtration of rat serum, normal (250 μ l) (o - o), and 15 min. post infusion of buffer containing taurocholate (250 μ l diluted 2:1) (● - ●). (For details; see Methods).

DISCUSSION

Previous authors have described the technique of taurocholate induced pancreatitis in the rat (1,2). It has been described both with and without the addition of trypsin to the bile-pancreatic duct taurocholate infusate. Aho and Nevalainen have recently used electron microscopy to study this model's mechanism of onset. They used the bile salt alone, and postulated that when performed properly, the progression of the disease is initially due to the detergent action of taurocholate on the acinar cell membranes and not simply due to the pressure of injection (2). It was their impression that later necrotic lesions develop as the result of the action of the native digestive enzymes.

A radioimmunoassay for rat trypsin has been described previously by Temler (8). However, it has not been used, to the best of our knowledge, to study serum levels of this enzyme.

The radioimmunoassay described for trypsin in this report was of sufficient sensitivity to allow us to detect circulating levels of trypsin-like immunoreactivity in the normal rat. Dilution curves of rat serum, pancreas juice, standard DFP inactivated anionic trypsin were essentially parallel, indicating that the same immunoreactive substance was being measured in each case.

Circulating trypsin, PSTI and α -amylase levels are considerably higher in the rat than in the human. In part this facilitates accurate measurements using small sample volumes. We found circulating levels of trypsin equivalent to approximately five times those of the human (9). The PSTI baseline level in this experiment was $18.8 (\pm 3.2) \mu\text{g/l}$ which is about twice the human level (10). α -Amylase levels were about 4500 units/l but represent a combination of salivary and pancreatic levels, as a non-specific enzymatic assay was used.

As in Aho's work, we eliminated trypsin from our duct infusate. We chose to do this so as not to induce direct external tryptic pancreatitis or to complex the intrinsic pancreatic trypsin specific inhibitor, PSTI. We found that serum levels of amylase

rose only slightly until 30-60 min. post infusion. At this point the control group began returning to normal levels, while a sharp rise was found in the taurocholate group. A significant difference between the control and experimental groups was apparent in most cases within 30 to 60 min. after duct infusion for both PSTI and trypsin. PSTI returned to baseline levels within 2-6 hours and had a maximum of 150 % of the baseline value at 30 min. Trypsin immunoreactivity was slow to return towards baseline levels in the controls and at 2 hours was still 250 % over baseline, and 160 % at 6 hours.

These findings tend to agree in part with those of Satake *et al.* who noted a significant increase in serum elastase immunoreactivity during pancreatitis over α -amylase activity. This may be explained in part by the fact that both of these molecules are serine endoproteases and therefore complexed by the native serum protease inhibitors, α -macroglobulin (α -M), α_1 -inhibitor₃ (α_1 -I₃) and α_1 -antitrypsin (α_1 -AT (11). Although α -M-bound proteases are probably rapidly eliminated in the liver by the Kupfer cells, α_1 -AT-bound proteases tend to have a longer circulating half life, and the complexed protease remains immunologically detectable (12). On the other hand, PSTI has been shown by ourselves and others to be rapidly eliminated from the circulation (5,13). α -Amylase (MW 45,000) likewise is rapidly eliminated by the kidney (14).

Gel filtration of normal rat serum resulted in both immunoreactive trypsin and PSTI being eluted in a volume identical to free trypsin and free PSTI, respectively. During acute pancreatitis amounts of these substances increased dramatically. Trypsin immunoreactivity was still eluted in a volume consistent with free trypsin, but also to a lesser extent, it was demonstrated in the volume of the serum protease inhibitors, indicating complexation of active enzyme by the serum protease inhibitors. A great deal of trypsin immunoreactivity was eluted in the elution volume of free trypsin as well as volumes of lesser molecular weight. This agrees with the observation of Borgström who noted that in acute pancreatitis not only was active trypsin liberated, but also large amounts of its inactive precursor,

trypsinogen (15). The second and therefore lower molecular weight peak of trypsin-like immunoreactivity and its extended shoulder may represent breakdown products of activated trypsin containing immunogenic sites.

PSTI immunoreactivity was also eluted in two peaks. The smaller of these peaks eluted at the volume of free PSTI, and the larger at about the elution volume of the column. This later peak indicates breakdown products of PSTI containing immunogenic sites. The latter observation is consistent with the findings of others that the PSTI molecule undergoes a series of hydrolyses in the presence of active trypsin, resulting in fragmentation of the inhibitor (16,17). This finding supports the assumption that trypsinogen is activated in this form of pancreatitis as well as Aho and Nevalainens' speculation that part of the pathophysiologic picture of this form of pancreatitis is due to enzyme activation.

Finally, following the initial duct infusion, serum levels of all three proteins had small sharp rises. The shape of the circulating proteins' curves over time is important. A relative plateau was reached for trypsin and α -amylase which lasted 15 to 30 min. in the taurocholate group, and was then followed by a second marked increase in their levels. This suggests that two phases of the disease exist in this model. The first is the insult due to the pressure of injection, forcing reflux into the circulation of the pancreatic proteins, and probably some acinar disruption with resultant edema and local ischemia. When the bile salt is present in the infusate a second phase then takes place, which involves the activity of the native digestive enzymes. Part of the reason for the delay in the onset of the second phase may be due to a latent period during which the taurocholate is acting on the cell membranes and inducing protease activation. It may also result from a need to overwhelm the locally produced trypsin inhibitor, PSTI, prior to triggering activation of the key pro-enzyme trypsinogen. This latter possibility is not unlikely as PSTI is produced in only about 1-2 % of the amount of trypsinogen (10).

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Studies on the Interaction of Trasylol (Aprotinin) with Trypsin-Pancreatic Secretory Trypsin Inhibitor (PSTI) Complexes and with α_2 -Macroglobulin-Trypsin-PSTI-Complexes

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Summary: An investigation was performed to study the interaction of Trasylol with both trypsin-pancreatic secretory trypsin inhibitor (PSTI) and α_2 -macroglobulin (α_2 -M)-trypsin-PSTI complexes. Trasylol was readily able to displace immunogenic PSTI from a complex with trypsin in vitro. A similar scale of displacement of PSTI

by Trasylol from α_2 -M-trypsin-PSTI complexes could not be demonstrated. Using complexes manufactured in vitro with ¹²⁵I-labelled PSTI, we found that only a small percentage of the PSTI label could be liberated, even when presented with amounts of Trasylol in a 10-molar excess to the PSTI.

Zur Reaktion von Trasylol (Aprotinin) mit Komplexen von Trypsin mit dem sekretorischen Pankreas-Trypsin-inhibitor (PSTI) bzw. mit α_2 -Makroglobulin-Trypsin-PSTI-Komplexen

Zusammenfassung: Es wurde die Reaktion von Trasylol mit dem Trypsin-PSTI-Komplex und mit dem α_2 -Makroglobulin-Trypsin-PSTI-Komplex untersucht (PSTI: pankreatischer sekretorischer Trypsininhibitor). Trasylol konnte den immunologisch nachweisbaren PSTI aus dem Trypsin-PSTI-Komplex rasch in vitro verdrängen, nicht jedoch aus dem α_2 -Makroglobulin-Trypsin-PSTI-

Komplex. Wenn in vitro hergestellte Komplexe mit ¹²⁵I-markiertem PSTI eingesetzt wurden, wurde nur ein geringer Prozentsatz des markierten PSTI aus den Komplexen freigesetzt, selbst wenn ein 10facher molarer Trasylolüberschuß (bezogen auf PSTI) verwendet wurde.

Key words: α_2 -Macroglobulin, pancreatic secretory trypsin inhibitor, Trasylol, trypsin.

In the pancreas, premature activation of trypsin and subsequent autodigestion of the gland are probably prevented by the low molecular mass pancreatic secretory trypsin inhibitor of Kazal-type (PSTI)^[1]. The

enzyme-inhibitor complex formed is temporary in nature and results in the release of modified inhibitor of reduced or no activity^[2,3]. The desire to attack a disease process on a molecular basis had led to the ex-

Enzyme:

Trypsin (EC 3.4.21.4).

Abbreviations:

α_2 -M = α_2 -Macroglobulin;

PSTI = pancreatic secretory trypsin inhibitor;

Bz = benzoyl; Nan = *p*-nitroanilide.

perimental use of Trasylol*, an exogenous low molecular mass trypsin inhibitor, for combatting severe clinical and experimental pancreatitis, a pursuit which has been met with mixed success^[4,5].

Previous investigators have demonstrated and characterized the release of PSTI from its complex with trypsin at acid pH *in vitro*^[2]. The interactions of α_2 -macroglobulin (α_2 -M), trypsin and PSTI *in vivo* and *in vitro* were recently studied in this laboratory, and PSTI was shown to be rapidly displaced from a complex with trypsin in serum^[6,7].

However, to date there are no reports in the literature which look at the interaction of Trasylol with trypsin-PSTI or α_2 -M-trypsin-PSTI complexes. The main purpose of this investigation was to determine if Trasylol is capable of displacing PSTI from complex with trypsin and/or α_2 -M-trypsin *in vitro*.

Materials

Sephadex G-25, G-50, G-75, G-200, and SP-Sephadex were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Agarose, batch 563, was a product of Miles Lab., U.S.A. Trasylol (Aprotinin) Lot K1/73, 5600 KIU**/mg, was a gift of Bayer AG, Leverkusen.

Cationic human trypsin was prepared from human pancreas juice as described by Ohlsson and Skudel^[8]. About 85% of the enzyme preparation was found to be active trypsin by active site titration^[9].

PSTI was isolated from human pancreatic juice according to Eddeland^[7]. The spec. activity was about 2.7 IU/mg protein (trypsin inhibition; substrate BzArgNan, E. Merck, Darmstadt).

Radioactive counting was done in a well-type scintillation detector produced by Nuclear Enterprises, Edinburgh, Scotland.

Protein concentration in gel filtration fractions was monitored by absorbance at 280 nm.

Antisera: Sheep anti-human cationic trypsin (titre: ~ 350 μ g/ml) and rabbit antihuman PSTI (titre: ~ 100 μ g/ml) are available in our laboratory^[7,8].

Methods

All procedures, unless otherwise indicated, were carried out at 4 °C.

* Trasylol (generic name Aprotinin) is identical with the basic pancreatic trypsin inhibitor (BPTI) Kunitz of bovine origin.

** Kallikrein inhibitor units.

Immunoradial diffusion was performed according to Mancini et al.^[10], using the appropriate antiserum in a dilution of 1/100–1/200.

¹²⁵I-labelling of PSTI and Trasylol was accomplished using a lactoperoxidase method to give a spec. activity of about 2 mCi/mg^[11]. Gel filtration on a Sephadex G-25 column (0.9 x 15 cm) equilibrated with Tris/HCl, 0.04M, pH 7.6, containing 0.002M CaCl₂ and 0.2M NaCl was used to separate unbound ¹²⁵I from labelled protein.

Radioimmunoassay for PSTI as described by Eddeland and Ohlsson^[12] is available as a routine procedure in our laboratory.

Preparation of PSTI-trypsin complex and displacement of PSTI from complex with trypsin

Release of PSTI from purified trypsin-PSTI complex by Trasylol: 200 μ g of active site titrated^[9] human cationic trypsin was incubated with about 100 μ g PSTI at room temperature for 30 min in a 500 μ l reaction volume of 0.04M Tris/HCl buffer, pH 7.6 containing 0.002M CaCl₂ and 0.2M NaCl. The mixture was then applied to a Sephadex G-75 column (0.3 x 60 cm) equilibrated with the same buffer. Samples of the eluted fractions were tested by radial immunodiffusion using anti-trypsin or anti-PSTI. An excess of PSTI was apparent. The first half of the trypsin-containing fractions was pooled and concentrated by ultrafiltration to 600 μ l and divided into 30 μ l aliquot portions. Following this, 30 μ l of the Tris/HCl buffer containing increasing amounts of Trasylol (0 to 15 μ g) was added. After 30 min incubation at room temperature, 10 μ l samples were screened using the Mancini technique with PSTI anti-serum.

α_2 -M-trypsin-PSTI ¹²⁵I-complexes were manufactured using a technique similar to that of Eddeland and Ohlsson^[13]. 5 ml of fresh human serum was gel filtered on Sephadex G-200 (2.5 x 40 cm) using the same Tris/HCl buffer as above for elution. The first half of the initial peak was pooled and concentrated to about 2.5 ml on a UM-10 Amicon membrane. Human cationic trypsin (250 μ g) was then added and allowed to react for 10 min at room temperature. The entire sample was then filtrated on the same column using the same buffer. A single major peak was eluted, the first half being pooled and concentrated to 3 ml on a UM-10 Amicon filter and incubated with ¹²⁵I-labelled PSTI (50–70 μ g) for 10 min at room temperature.

The mixture was then gel filtrated again using the same system. The initial radioactive peak corresponding to the elution volume of α_2 -M was pooled and concentrated by ultrafiltration to 3 ml.

Displacement of ¹²⁵I-PSTI from α_2 -M-trypsin-PSTI ¹²⁵I complex. An aliquot portion of the complex

mentioned above was incubated for 30 min at room temperature with Trasylol in a ten-fold molar excess compared to the available trypsin. The reaction medium consisted of 5 ml of the Tris/HCl buffer. The entire reaction mixture was then charged to the Sephadex G-200 column and the radioactivity of the eluted fractions was measured in a scintillation detector of well type.

Results

I. Trypsin-PSTI complex + Trasylol

Release of PSTI from purified trypsin-PSTI complex

Our antiserum for PSTI is known to react exclusively with uncomplexed PSTI^[6,7]. The addition of increasing amounts of Trasylol, 0 to 15 μ g, to the complex resulted in the release of increasing amounts of immunogenic PSTI, as determined by the Mancini technique (Fig. 1). In the final reaction mixture about 80–90% of trypsin bound PSTI was released.

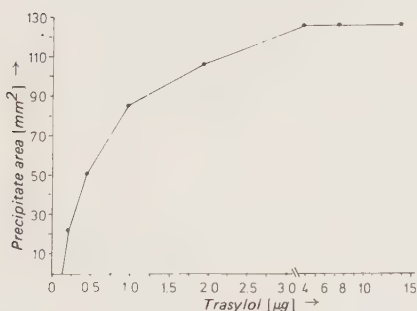


Fig. 1. Release of PSTI from complex with trypsin by the addition of Trasylol.

Plot of the areas of precipitates of PSTI-anti-PSTI complex obtained in radial immunodiffusion, resulting from the addition of increasing amounts of Trasylol (0–15 μ g) to constant amounts of trypsin-PSTI complex containing ~ 2 μ g of PSTI. (See Methods, for details).

II. Release 125 I-labelled PSTI from α_2 -M-trypsin- 125 I-PSTI complex

The addition of Trasylol in about 10 times the molar concentration of the available trypsin caused a release of approximately 20% of the PSTI label. The displaced label appeared in two peaks, the first (a) corresponding

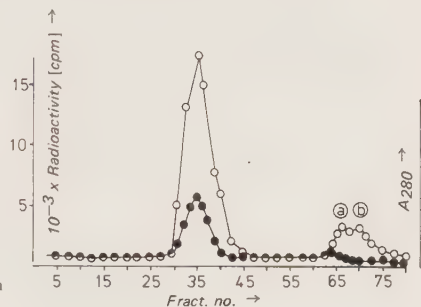


Fig. 2. Release of 125 I-PSTI by Trasylol from α_2 -M-trypsin- 125 I-PSTI-complex.

Gel filtration on Sephadex G-200 of α_2 -M-trypsin- 125 I-PSTI complex containing approximately 200 μ g available α_2 -M-bound trypsin following incubation at room temperature for 15 min with 1 mg of Trasylol. $\sim 20\%$ of the counts were displaced and found in two peaks. One (a) corresponding to the elution volume of PSTI with about 10% of the total counts and the second (b), unidentified one containing additional 10%. (Column size: 2.5 $\times$ 40 cm. Fraction volume: 2.5 ml) l.

○—○ = cpm ●—● = A_{280} .

to the elution volume of PSTI and the second (b) is as yet unidentified (Fig. 2).

Discussion

Previous investigators have reported that α_2 -M-bound trypsin is capable of activating trypsinogen at about 50% of the rate of free trypsin^[14]. The suppression of tryptic activity is one goal of utilizing Trasylol in acute pancreatitis. Although it has been demonstrated that PSTI, in a modified form, is released from trypsin at low pH, the ability of Trasylol to displace PSTI from trypsin has not been demonstrated yet. Neither has Trasylol's interaction with α_2 -M-trypsin-PSTI complex been studied to date. These interactions need to be elucidated, however, in order to objectively judge the roles of Trasylol and PSTI in acute pancreatitis.

Previous reports from this laboratory have demonstrated the rapid dissociation of the complex of 125 I-labelled PSTI and 131 I-labelled trypsin when placed in serum in vitro and in vivo in both man and dog^[7].

The present investigation demonstrates that Trasylol readily displaces unlabelled PSTI from the complex with unlabelled trypsin in vitro.

However, we were unable to demonstrate a similar marked release of ^{125}I -PSTI from a complex with α_2 -M-bound trypsin. Only approximately 20% of the label was displaced by Trasylol, even when Trasylol was present in high molar excess over the available trypsin. The released label was found in two small peaks, each representing about 50% of the total counts. The first peak was eluted in a volume corresponding to PSTI. The second, immediately following peak corresponds to a smaller molecular size, thus suggesting formation of a degradation product.

In summary, we have shown that human PSTI is readily displaced by Trasylol from its complex with human trypsin but only to a limited extent when bound to human α_2 -M-trypsin complex. These findings may be useful for understanding molecular interactions of Trasylol with endogenous inhibitors and proteases in vivo, especially as they apply to acute pancreatitis.

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ON THE ROLE OF THE PANCREATIC SECRETORY TRYPSIN INHIBITOR AS AN INACTIVATOR OF TRYPSIN - ALPHA - 2 MACROGLOBULIN COMPLEXES IN ACUTE PANCREATITIS

* * ** *

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High levels of immunoreactive pancreatic secretory trypsin inhibitor (PSTI) were demonstrated in the serum and peritoneal exudates of patients having acute pancreatitis. Trypsin-like immunoreactivity in these fluids was found in complex with α_1 -antitrypsin and in complex with α_2 -macroglobulin as well as a free peak correlating to free trypsin(ogen). No trypsin-PSTI complexes or PSTI in the macroglobulin fraction of the peritoneal exudates were demonstrated. Saturated and partially saturated trypsin- α_2 -macroglobulin complexes are prepared in vitro. PSTI was able to partially inhibit the BAPNA-cleaving activity of both types of complexes in a slow dose-dependent non-linear reaction. Equilibrium was reached in each case within 1 hour but total inhibition was not reached even with large amounts of PSTI. Partially saturated trypsin- α_2 -macroglobulin complexes were inhibited more readily than saturated complexes.

Patients suffering from severe acute pancreatitis have about 10 % of the α_1 -antitrypsin in their peritoneal exudates in complex with pancreatic proteases (1). Furthermore, over 50 % of the α_2 -macroglobulin of these exudates is in a complexed form. Similarly, increased concentrations of immunoreactive trypsin have been demonstrated in serum, part of it identified as trypsin- α_1 -antitrypsin complexes (2), supporting the concept of a pronounced activation of trypsin in acute pancreatitis.

The pancreatic secretory trypsin inhibitor (PSTI) is believed to act as a local protease inhibitor protecting the pancreas against the deleterious effects of prematurely activated trypsin. In a serum-like milieu PSTI-bound trypsin is rapidly given up to α_2 -macroglobulin (3,4). Residual hydrolytic activity against low molecular weight substrates shown by trypsin in complex with α_2 -macroglobulin has been shown in one study to be inhibited *in vitro* by PSTI (5). More recently we have been impressed by our ability to incorporate only minimal amounts of PSTI into human α_2 -macroglobulin-trypsin complexes formed *in vitro* (6).

The purpose of the present study was to further elucidate the role of PSTI as an inhibitor of the trypsin- α_2 -macroglobulin complexes formed *in vivo* in man during acute pancreatitis by: a) Measuring and characterizing immunoreactive PSTI-levels in sera and peritoneal exudates collected from patients having acute pancreatitis, b) Determining the PSTI content of trypsin- α_2 -macroglobulin complexes in peritoneal exudates collected from patients having acute pancreatitis and c) Studying the ability of PSTI to inhibit both partially and fully saturated trypsin- α_2 -macroglobulin complexes formed *in vitro*.

MATERIALS AND METHODS

Sephadex G-100 and G-200 were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Agarose was obtained from Miles-Seravac, Maidenhead, England;

Benzoyl-DL-arginine-p-nitroanilide-HCl (BAPNA) was a product of Fluka AG, Switzerland.

Cathodal human trypsin (7) and the human pancreatic secretory trypsin inhibitor (PSTI) (4) were purified as previously described.

Antisera against human α_2 -macroglobulin, α_1 -antitrypsin and PSTI

are available in our laboratory.

Tryptic activity and trypsin inhibiting capacity were determined with BAPNA as substrate according to Erlanger *et al* (8).

Immunochemical method

Electroimmunoassay (9) and crossed immunoelectrophoresis (10) were used for identifying α_2 -macroglobulin, α_1 -antitrypsin and their complexes with trypsin.

Radioimmunoassays for PSTI (11) and trypsin (12) are available in our laboratory.

Clinical material

During a 5-year period (1976-1980), 28 patients with severe acute pancreatitis were treated with peritoneal lavage at the Department of Surgery, Malmö General Hospital. All patients had abdominal tenderness with diffuse or bilateral upper quadrant muscular rigidity. The patients were judged to need admission to the intensive care unit. Peritoneal exudates were recovered from each of 15 patients who had dark, red-brown hemorrhagic exudates in large amounts. In addition, a specimen of venous blood was drawn simultaneously from all patients into test tubes containing 10 mg EDTA. Plasma and supernatants recovered after centrifugation of the samples were either examined immediately or stored at -20°C .

Preparation of partly ($\sim 50\%$) and fully saturated trypsin- α_2 -macroglobulin complexes

Increasing amounts of human trypsin (0-60 μg in 0.1 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl) were added to fresh human serum. The reaction mixtures were made up to a volume of 200 μl with the Tris-HCl buffer and incubated at room temperature for 15 min before the addition of 500 μl BAPNA solution. Trypsin activity was determined after 30 min of incubation at room temperature by measuring absorbance at 405 nm (18). This absorbance curve was used to determine the amount of trypsin necessary for 50 % and fully saturated trypsin- α_2 -macroglobulin complexes according to Ganrot (13).

Based on these titrations, reaction mixtures were prepared using 1 ml of fresh human serum and human cathodal trypsin sufficient to give 50 % and 100 % saturation, respectively, of the α_2 -macro-

globulin present. Reaction mixtures were then applied to Sephadex G-200 columns (2.5 x 40 cm), equilibrated with 0.01 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. The flow rate was 7.5 ml/h. Fractions were 4.0 ml. α_2 -Macroglobulin-trypsin complexes were identified using electroimmunoassay for α_2 -macroglobulin and BAPNA-cleaving activity. The peak fractions of each run were pooled for further analyses.

Inhibition of partly (50 %) and fully saturated trypsin- α_2 -macroglobulin complexes with PSTI

In separate experiments increasing amounts of PSTI (1.0 mg/ml) dissolved in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl were added to 80 μ l of the partly or fully saturated trypsin- α_2 -macroglobulin fractions. Final volume was made up to 500 μ l with Tris-HCl buffer. The reaction mixtures were incubated for 10, 60 and 240 min at +37 °C. Trypsin inhibiting capacity was then determined using BAPNA as substrate (8).

Identification of trypsin and PSTI in PSTI-trypsin- α_2 -macroglobulin formed *in vitro*

PSTI was added to partly and fully saturated trypsin- α_2 -macroglobulin complexes to give 25 % inhibition of the BAPNA-cleaving activity. The reaction mixtures were incubated at 37 °C for 60 min and then applied to a Sephadex G-200 column (0.9 x 60 cm), equilibrated with 0.1 M Tris-HCl buffer, pH 7.4, containing 0.5 M NaCl. The flow rate was 1.5 ml/h and the effluent was collected in 500 μ l fractions. The α_2 -macroglobulin-containing fractions were pooled. Half of this amount (500 μ l) was incubated with 250 μ l of the same buffer, as mentioned above, for 24 hrs at room temperature. The other half was incubated at the same time and at the same temperature with 0.2 M formic acid, pH 2.5. The samples were subjected to gel filtration on a Sephadex G-100 column (0.9 x 60 cm), equilibrated with the Tris-HCl buffer and with 0.2 M formic acid, pH 2.5, containing 0.5 M NaCl. The fractions were analyzed for cathodal trypsin and PSTI.

Identification of trypsin and PSTI-trypsin- α_2 -macroglobulin complexes in peritoneal exudates

Five milliliters of peritoneal exudate from each patient were applied to a Sephadex G-100 column (2.5 x 40 cm), equilibrated with 0.1 M Tris-HCl buffer, pH 7.4, containing 0.5 M NaCl and 0.005 M EDTA. The flow rate was 20 ml/h and the effluent was collected in 4.0 ml fractions. α_1 -Antitrypsin and α_2 -macroglobulin were identified using electroimmunoassay. Trypsinogen, trypsin in complex with α_1 -antitrypsin and PSTI were identified using radioimmunoassay. The α_2 -macroglobulin containing fractions were pooled and concentrated to 2 ml by ultrafiltration. One milliliter was incubated for 24 hrs at room temperature with 1.0 ml of the Tris-HCl buffer at the same temperature with 1 ml of 0.2 M formic acid (pH 2.5). The samples were subjected to gel filtration on a Sephadex G-100 column (2.5 x 40 cm) at a neutral and acid pH, respectively. All fractions were analyzed for cathodal trypsin and PSTI using radioimmunoassay.

RESULTS

The pancreatic secretory trypsin inhibitor (PSTI) in peritoneal exudates and serum from patients with severe acute pancreatitis

In the peritoneal exudates from 15 patients with severe acute pancreatitis, the mean value of PSTI was 180 $\mu\text{g/l}$, range 17-350 $\mu\text{g/l}$. The corresponding initial values in serum from 28 patients were 155 $\mu\text{g/l}$, range 5-800 $\mu\text{g/l}$. The highest value noted in serum during the treatment was 7,500 $\mu\text{g/l}$. Fig. 1 shows distribution of proteins after separation of peritoneal exudate on Sephadex G-100. α_2 -Macroglobulin and α_1 -antitrypsin eluted as a single peak each, while immunoreactive trypsin eluted in two peaks corresponding to trypsinogen or free trypsin and trypsin- α_1 -antitrypsin complexes. PSTI was consistently identified as a rather broad peak eluting roughly at the size of free inhibitor.

Inhibition by PSTI of trypsin- α_2 -macroglobulin complexes formed *in vitro*

PSTI blocked the BAPNA-cleaving activity of trypsin- α_2 -macroglobulin complexes to a limited extent (Fig. 2). Even in the presence

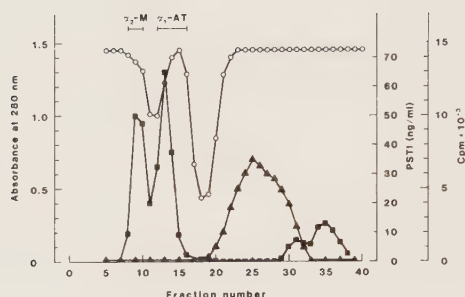


Fig. 1. Partition of α_2 -macroglobulin, α_1 -antitrypsin, immunoreactive trypsin and immunoreactive PSTI from gel filtration on Sephadex G-100 of 5 ml of peritoneal exudate from a patient with severe acute pancreatitis. (For details; see methods).

of large excesses of PSTI the half saturated complexes could be inhibited to only $\sim 40\%$ of base line activity and the fully saturated complexes to $\sim 60\%$. Equilibrium was reached after 60 min in both cases.

Identification of PSTI and trypsin in α_2 -macroglobulin complexes formed *in vitro* and in peritoneal exudate

No PSTI or trypsin immunoreactivity was demonstrable after re-gel filtration of the macroglobulin fractions incubated at neutral pH. However, following acidification immunoreactive trypsin was detected in the re-chromatographed macroglobulin fractions from all patients. Trypsin immunoreactivity separated into two protein peaks, one corresponding in size to free trypsin, while the other was of higher molecular weight (Fig. 3). PSTI immunoreactivity was demonstrated in the macroglobulin fraction of only one patient after acidification. On the other hand, acidification of *in vitro* prepared PSTI-trypsin- α_2 -macroglobulin complexes consistently contained PSTI and trypsin-like immunoreactivity.

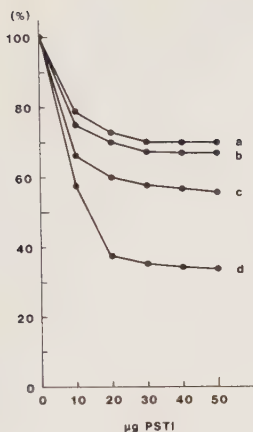


Fig. 2. Titration of the trypsin-like activity of semi-saturated and saturated trypsin- α_2 -macroglobulin complexes with PSTI. Substrate used in the test-system: BAPNA.

(a) Saturated trypsin- α_2 -macroglobulin complexes after 10 min pre-incubation with varying amounts of PSTI, (c) the same complexes after 60 min of pre-incubation, (b) semi-saturated complexes after 10 min pre-incubation with varying amounts of PSTI and (d) the same complexes after 60 min pre-incubation, Pre-incubation of the complexes with PSTI for 240 min resulted in identically inhibition curves.

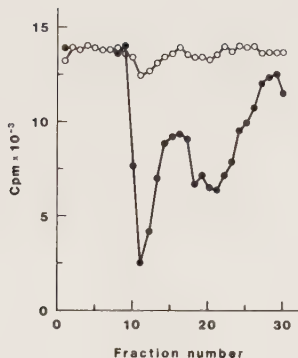


Fig. 3. Partition of immunoreactive trypsin on gel filtration of acidified and non-acidified macroglobulin fractions from peritoneal exudates of patients with severe acute pancreatitis on Sephadex G-100.

1 ml of macroglobulin fraction was incubated at 24 hrs at neutral pH ○ - ○ and 1 ml from the same macroglobulin fraction was incubated at pH 2.5 for 24 hrs before separation ● - ●

DISCUSSION

Increased levels of PSTI were consistently demonstrated in the serum and peritoneal exudates of patients with severe acute pancreatitis. PSTI was only present in its free form in the peritoneal exudates. As large amounts of the serum protease inhibitors are also present in these exudates, this finding is in agreement with earlier experimental results showing rapid cleavage of trypsin and PSTI complexes in the presence of plasma protease inhibitors (4).

Half saturated trypsin- α_2 -macroglobulin complexes were inhibited more readily by PSTI than fully saturated trypsin- α_2 -macroglobulin complexes. Similar inhibitory interactions have been demonstrated for aprotinin (14), soybean trypsin inhibitor (15) and antileukoprotease (16). Bieth has hypothesized that this difference in inhibition between partially and fully trypsin saturated α_2 -macroglobulin complexes is the result of stearic induction (15). Regardless the inhibition rate is remarkably slow, taking from about 1 hour to reach equilibrium for the lower molecular weight inhibitors, PSTI and aprotinin (MW $\sim$ 6,200) (14) and 12 hours for soybean trypsin inhibitor (MW $\sim$ 18,000) (15). Antileukoprotease (MW $\sim$ 10,000) comes to equilibrium between these extremes (16).

Trypsin- α_2 -macroglobulin complexes were consistently demonstrated in the peritoneal exudates of these patients and free PSTI was always present although no immunoreactive PSTI could be identified in the α_2 -macroglobulin fraction. In support of this the macroglobulin fractions from peritoneal exudates in experimental (17) and severe clinical pancreatitis (18) have shown considerable trypsin-like activity. It would seem desirable if a low molecular weight inhibitor was capable of blocking the proteolytic activity of α_2 -macroglobulin-bound trypsin as previous authors have demonstrated potentially harmful effects of these complexes such as *in vitro* activation of trypsinogen (19, 20) and degradation of the parathyroid hormone (21). However, this type of activity may actually prove to be without consequence as more recent work both *in vivo* and *in vitro* indicates that the

complement (22,23) and kallikrein-kinin (24,25) effector systems are not activated by these complexes. As important, are experimental studies which have demonstrated proteolytic activity of these trypsin- α_2 -macroglobulin complexes only after long incubation periods (19). *In vivo* protease- α_2 -macroglobulin complexes are rapidly cleared from the circulation ($t_{1/2}$ 5-10 min) (26,27) and free macrophages *in vitro* have been shown to clear these complexes with a half time of 15 min (28). Although the *in vivo* data on the elimination of peritoneal protease- α_2 -macroglobulin complexes is lacking large numbers of macrophages are known to exist in the peritoneal cavity.

Therefore, in conclusion, the data from the present study in light of prior evidence argues strongly against a role for PSTI as an inhibitor of trypsin- α_2 -macroglobulin complexes *in vivo*.

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THE ELIMINATION OF PANCREATIC SECRETORY TRYPSIN INHIBITOR FROM THE CIRCULATION A STUDY IN MAN

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An investigation into the elimination of human pancreatic secretory trypsin inhibitor (PSTI) from the circulation of man has been conducted. Two human volunteers were utilized. Serial samplings of blood and urine were made for 55 hours following a rapid intravenous infusion of 125 -labelled human PSTI.

The findings demonstrated a rapid initial elimination from the circulation. Within 30 min only 30 % of the infused label remained ($T_{1/2}$ 6 min). This was accompanied by the rapid appearance in the urine of radiolabel.

*Our results indicate that PSTI should prove a poor diagnostic marker for acute pancreatitis late in the course of the disease. This is opposed to the findings of Ogawa *et al* who reported prolonged elevated circulating levels of PSTI weeks into the disease. However, they also noted rises of PSTI during acute pancreatitis in excess of 10 times the levels we have noted.*

Further exploration into population differences and the behavior of PSTI during acute pancreatitis are necessary to help resolve these findings.

Key-words: Pancreatitis; trypsin; trypsin inhibitors

The pancreatic secretory trypsin inhibitor of Kazal (PSTI) (mw 6200) has undergone analysis in both man (1,2) and animals (3,4,5,6) in attempts to define its roles in normal and pathophysiological processes of the pancreas. The behavior *in vitro* of PSTI was recently studied by Eddeland and Ohlsson (7,8). They described the elimination of radiolabelled PSTI from the circulation of dogs (7). Their results correlated with the findings of others concerning another low molecular weight trypsin inhibitor, aprotinin (mw 6300).

Recently Ogawa *et al.* have reported their findings concerning serum levels of PSTI during acute pancreatitis in man (9). In their study average peak levels of $795 \pm 1084.2 \mu\text{g/l}$ were noted. They also noted that serum levels of PSTI remained elevated for weeks into the disease and were more sensitive than amylase in following its clinical course. Our radioimmunoassay (RIA) (10) and that recently reported by Ogawa *et al.* (11) have remarkably similar properties. However, in a preliminary review of our own clinical studies concerning PSTI in acute pancreatitis, we have found average serum levels of only $138.8 \mu\text{g/l}$ (12). In order to better assess the potential role of PSTI as an indicator of acute pancreatitis, we felt an investigation into the elimination pattern in the human was warranted.

The purpose of this project was to study the elimination of ^{125}I from the circulation of human volunteers.

MATERIALS AND METHODS

PSTI was purified as previously described (3).

Radiolabelling of PSTI: Approximately 250 μg of PSTI was labelled with $4.25 \text{ MBq } ^{125}\text{I}$ using a lactoperoxidase technique (13). Free ^{125}I and lactoperoxidase were separated from the labelled material by gel filtration on a Sephadex G-50 column (0.9 x 50 cm) using a 0.01 M Tris-HCl buffer, pH 7.4 and 0.2 M NaCl. 1.4 ml fractions were collected and aliquots of all fractions subjected to γ -counting in a well-type scintillation counter. For the first experiment the peak fraction, totaling about 89 million cpm, was used. For the second experiment the fractions immediately before

and after the peak fraction were pooled and 75 % of these counts were used to give a total of approximately 86 million counts per minute.

The PSTI retained its biologic activity after labelling as shown by measurements of trypsin inhibiting capacity of the labelled protein after gel filtration as detailed before (14). There was no release of free ^{125}I on incubation of the labelled PSTI in serum/plasma for 4 hours.

Volunteers: Two healthy female volunteers, aged 27 and 29 years, were used. Both gave informed consent. Each was begun on an iodine preparation (Draco AB, Lund) prior to the experiment.

A large bore indwelling intravenous catheter was placed in a peripheral arm vein. Following baseline blood sampling, ^{125}I -PSTI was infused in a total volume of 3.5 ml over 30 seconds. Sampling began 10 min. later and 4 ml samples were drawn every 10 min. for 3 hours. Additional samples were taken at 24 and 48 hours. Serum was immediately removed from all samples and 1 ml aliquots were used for γ -counting.

Urine: The volunteers were asked to void immediately preceding the infusion of ^{125}I -PSTI. They were then instructed to void as frequently as was feasible over the next 50-55 hours. Volumes of each void were recorded and 1 ml aliquots were counted.

Gel filtration: was performed on 0.9 x 60 cm Sephadex G-50 columns equilibrated and eluted with 0.05 M Tris-HCl, pH 7.4, containing 0.02 M NaCl and 0.01 M CaCl_2 . Fractions were 0.7 ml and the rate was 8 ml/hour. Serum samples from 10, 30, 45, 60 and 180 min. and urine samples from 6 and 18 hours were gel filtered. Aliquots of all fractions were subjected to γ -counting.

RESULTS

Absolute levels of circulating radioactivity fell rapidly. During the first 30 min. the $T_{1/2}$ was 6 min. and total counts fell to 25-30 % of the initial value. Following this initial drop, the rate of fall decreased. By 24 hours only 2 to 4 % of the initial dose remained, and by 48 hours the value was roughly 0.2 to 0.4 % (Fig. 1). Serial gel filtrations of serum samples revealed a rapid disappearance of free inhibitor as judged by a decrease in counts at the elution volume of free PSTI (Fig. 1). The disappearance of the free PSTI peak was accompanied by the appearance of radioactivity eluted in a volume larger than that of free PSTI.

Radioactivity rapidly appeared in the urine. Within 3 hours, the period of serum sampling, 25 % of the total injected counts appeared in the urine. By 12 hours about 75 % of the label could be accounted for in the urine, and by 24 hours 85-90 % was recovered (Fig. 2).

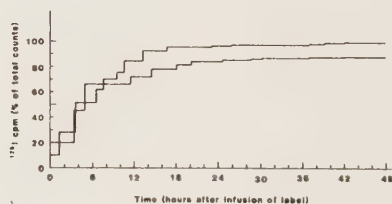
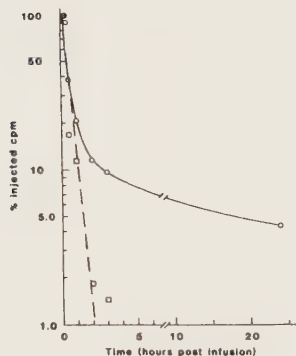


Fig. 1. Semi-log plot of per cent remaining gamma counts in 1 ml aliquots of serum taken from 10 min to 24 hours post infusion of ^{125}I -labelled PSTI (o-o). Also shown is a plot of peak heights of the free PSTI fractions in serial gel filtrations of serum following infusion of ^{125}I -labelled PSTI. Heights were determined as cpm in 1 ml aliquots as related to the 10 min sample (100 %) ($\square - \square$).

Fig. 2. Accumulation of ^{125}I in the urine of 2 human volunteers over 48 hours following infusion of ^{125}I -PSTI.

Gel filtration of aliquots of 6 and 18 hour urines resulted in one major radioactive peak eluting at the column volume. The 6 hour urine contained a greater concentration of activity than did the 18 hour sample. Also, a very small peak coming at the volume of free PSTI was present in the 6 hour sample (Fig. 3).

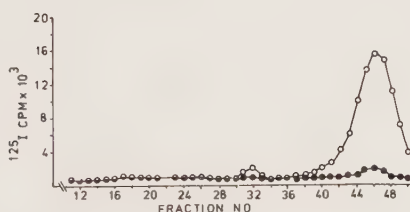


Fig. 3. Sephadex G-50 gel filtration of urine samples taken 6 hours (o - o) and 18 hours (● - ●) post infusion of ^{125}I -labelled PSTI. Free PSTI was eluted in fraction 32.

DISCUSSION

The elimination of free PSTI from the circulation of man is of importance in considering its usefulness as a diagnostic and/or prognostic indicator of acute pancreatitis. This is especially true since the recent report of Ogawa *et al.* who found PSTI to be a sensitive and prolonged indicator of acute pancreatitis, moreso than trypsin or amylase (9).

Eddeland *et al.* previously reported an elimination study of free PSTI in dogs (7). They described a rapid initial decrease of labelled free PSTI from the circulation ($T_{1/2}$ 8 min.) followed by a much slower decent ($T_{1/2}$ 24 hours). Similar findings in dogs were reported by Trautschold *et al.* for another low molecular weight inhibitor, aprotinin (15).

Our results agree with these previous authors concerning the clearance of this type of molecule from the circulation. Within

30 min. there was a rapid fall to less than about 30 % of the 10 min. value. The half life of labelled inhibitor in this initial phase was about 6 min. A prolonged decline then followed, having a half life of approximately 16 hours.

The half-life of the radiolabelled PSTI is judged to reflect that of unlabelled PSTI as the biologic function of the labelled and unlabelled PSTI, respectively, was identical. Furthermore, the labelling proved stable in a serum/plasma milieu *in vitro*.

Gel filtration studies of multiple serum samples revealed the rapid disappearance of free inhibitor as noted by a loss of counts in the elution volume of free PSTI. Likewise, no counts were eluted in a volume before that of free PSTI, indicating that PSTI circulates in its free form. This agrees with previous work (1). When serial serum samples were gel filtrated, γ -counts in the free PSTI volume rapidly disappeared. This was accompanied by the appearance of counts at the elution volume of the column. This seems to most likely represent the appearance of catabolic products of the inhibitor as it is rapidly metabolized.

Serial urine sampling demonstrated greater than 75 % of the injected counts in the urine within 12 hours of infusion. Gel filtration of these samples revealed the vast majority of counts appeared in a peak coming later than that of free PSTI. In the 6 hour sample a small amount of radioactivity was noted at the elution volume of free PSTI. This peak was missing at 18 hours.

Our results have demonstrated a rapid elimination of PSTI from the circulation in the human, which agrees with previous studies in lower animals using similar types of molecules(7,15). It seems likely that the rapid appearance of label in the urine results from its catabolism being, at least in part, in the kidney. This means of metabolism is as expected for free proteins under 50,000 mw (16).

The rapid elimination of PSTI from the circulation makes it an unlikely diagnostic indicator for acute pancreatitis late in the course of the disease. The possibility that PSTI exists in complex with activated trypsin and is therefore eliminated more slowly than free PSTI has already been indirectly addressed by previous work from this laboratory. Eddeland demonstrated that only a

small fraction of PSTI remains in complex with trypsin in a serum type milieu. Trypsin is given up to α_2 -macroglobulin and α_1 -antitrypsin leaving PSTI in its free form (7). Likewise, we have also demonstrated that immunodetection of PSTI is markedly impaired in our system of analysis when in complex with trypsin (14). Ogawa *et al.* reported a considerable loss of immunodetectable PSTI in complex with trypsin (11), again attributed to stearic hinderance of a major immunogenic site by trypsin. However, despite this and the remarkable similarity between our PSTI radioimmunoassays, Ogawa *et al.* have found PSTI to be a more sensitive late chemical indicator of acute pancreatitis than either trypsin, pancreatic elastase 1 and 2, or amylase. Their findings may relate to the massive (> 1.0 mg/l) increase of PSTI noted in their population during acute pancreatitis. Elevation of PSTI to similar levels has not been observed in our clinical material. Such high levels may result in prolonged overloading of PSTI's organ of elimination. The possibility that this elevation discrepancy is due to population variability or dietary induction also needs further investigation.

Continued study into the clinical behavior and usefulness of PSTI is currently underway in our hospitals.

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ENDOSCOPIC COLLECTION OF PURE PANCREAS JUICE FOR EXPERIMENTAL ANALYSIS: A SIMPLE PROTOCOL FOR ROUTINE USE

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A protocol is described which allows for the collection of pure pancreas juice for experimental study during routine endoscopic retrograde pancreaticography (ERP). Radio-contrast media can be used before sampling (Cerebral 280) thus simplifying the technique. The sampling adds less than 15 minutes to the morphological study. The influence of radio-contrast material on the immunochemical analytic techniques of radialimmunodiffusion (RID) and radio-immunoassay (RIA) as well as on an enzymatic technique for α -amylase was studied. No detrimental effects of the radio-contrast material were observed on pancreas juice or on the qualitative and quantitative characteristics of the analytic methods. This was true even when trace amounts of radio-contrast were present in stored inactive pancreas juice for periods extending 1 year.

Key-words: Amylase; cholecystokinin; pancreas secretin; secretion trypsin; trypsin inhibitors

Endoscopic Retrograde Pancreaticography (ERP) is a powerful clinical diagnostic technique which can also serve as a means of collecting pure pancreas juice samples for experimental studies. Its routine use for this purpose during clinical studies has been hindered by the lack of an easily performed protocol. Difficulty in developing a protocol relates in part to the use of radio-contrast material prior to pancreas juice collection. The use of radio-contrast media greatly facilitates selective cannulation of the main pancreatic duct, and is necessary for the requisite morphological studies. The effect of this material on the pancreas juice and on several techniques used to analyze pancreas juice components has not been described.

The purpose of this study was to establish a protocol for the collection of human pancreas juice on a routine basis, during clinically indicated ERP. We felt this protocol should fit the following criteria:

1. not interfere with the requisite morphological studies for which the patient was referred;
2. not interfere with the quantitative analytic methods necessary to evaluate the juice, or with the juice itself;
3. be simple, easily standardized, and performed at routine ERP without adding significantly to the time of the procedure;
4. not add to the morbidity of the study or discomfort of the patient.

MATERIALS AND METHODS

Protocol: Between December, 1979 and June, 1981, 84 patients undergoing ERP for a variety of complaints, underwent a selective pancreas duct cannulation followed by i.v. bolus secretin/cholecystokinin (Secretin/CCK-PZ) stimulation and pancreas juice collection. Of these, successful juice collections were obtained from 71 patients. Fourteen of these 71 patients had normal findings in the pancreas and no history of documented chronic or acute illnesses. Six of these 14 underwent their studies without the use of a pre-stimulation contrast injection. The remaining 8 underwent their studies following a selective

injection into the pancreatic duct of contrast material. Stimulation to aid juice flow and assure maximum secretory protein content was intravenous secretin and cholecystokinin (KABI) infused over 1 minute (1 U/kg of each to a maximum of 75 U). Pre-medication was atropine and droperidol followed by diazepam as needed, all given intravenously. None of the 14 patients in this group received glucagon or other antiperistaltic medications. All collections were made in 3 periods marked from time zero (the end of the secretin/CCK-PZ infusion):

Period I = 0 - 3.5 min

Period II = 3.5 - 9 min

Period III = 9 - 10 min post stimulation

These periods were chosen based on the work of Eddeland and Wehlin who demonstrated peak protein output and flow of juice coming approximately 5 to 7 minutes post i.v. infusion of secretin and cholecystokinin in human volunteers undergoing (ERP) (1).

Pancreas juice was collected on dry ice for transport and stored at -20°C . The samples in this study were in storage for 6 months to 1 year pre-analysis. Contrast media, when used, was Isopaque Cerebral 280 (Nyegaard & Co., Stockholm). All studies were performed using an Olympus side-viewing duodenoscope.

α -Amylase activity: was determined using the Phadebas technique as suggested by the manufacturer (Pharmacia Fine Chemicals, Uppsala, Sweden).

Benzoyl-DL-arginine-p-nitroanilide-HCl (BAPNA): (Fluka AG) was used as a trypsin specific substrate (2).

Tryptic activity of the juice was determined by incubating 100 or 200 μl of sample in a total of 2 ml Tris-HCl buffer, (0.05 M, pH 7.4) and 1 ml of BAPNA solution (1.3 mg/ml). The reaction was stopped using 30 % HAc (1 ml) and the absorbance determined at 410 nm (2).

Radiographs were made of collections taken from period 1 and 2. The tubes contained undisturbed frozen samples. Twenty-four samples were drawn at random from 65 cases in which collections were performed following a contrast-media pre-injection.

Antisera and radioimmunoassays for human cathodal trypsin (3),

pancreatic secretory trypsin inhibitor (PSTI) (4), and pancreatic elastase (PE) (5) are available in our laboratory on a routine basis. Each juice specimen was submitted for analysis following appropriate dilutions. As it was obvious following radial-immunodiffusion (RID) and the radiographs (see results) that only period 2 collections were worth analysis, radioimmunoassays (RIA) were performed on period 2 collections.

Radialimmunodiffusion (RID) was performed according to Mancini (6) using a 1% agarose gel with 3% polyethylene glycol added, as well as the appropriate antiserum.

In the first experiment, 10 μ l aliquots of each sample were allowed to diffuse 24 hours at 4 $^{\circ}$ C. The plates were washed extensively in normal saline and then pressed, dried and stained with Coomassie G-250. The areas of each immunoprecipitate were determined on the basis of direct observation through a calibrated eye piece.

In a second experiment, 500 μ l aliquots of inactive pancreas juice were incubated at room temperature for 30 min with increasing amounts of contrast material (0, 1, 10, 25 and 50 μ l). The final reaction volume was adjusted to 550 ml with 0.9 % NaCl. Ten μ l aliquots of each mixture were then subjected to RID as described above and the areas of each precipitate determined. Pancreas juice activity: Aliquots of pancreas juice without tryptic activity were incubated with increasing amounts of contrast media for 30 min at room temperature. BAPNA was then used to test for tryptic activity. Aliquots of all stored materials were also tested for tryptic activity.

Statistical analyses were performed using the Student's t test for unpaired data.

RESULTS

Effect of contrast media on pancreas juice: All inactive pancreas juice specimens remained inactive following incubation with contrast media. Radiographs of the randomly selected tubes demonstrated contrast material in 19 of 24 specimens. Varying amounts were present as is illustrated in Fig. 1. Trypsin-inactive pancreas juice specimens collected during the protocol lacked BAPNA activity following storage of up to 1 year at -20°C .



Fig. 1. Roentgenographs of randomly selected pancreas juice specimens collected during period 2 (3.5 - 9 min) post i.v. stimulation with secretin/CCK-PZ. All patients in this example underwent their studies having an injection of contrast media prior to stimulation. Contrast media is apparent in varying amounts.

Radialimmunodiffusion studies. In the first period the 6 patients studied without a contrast pre-injection demonstrated an average output of trypsin (expressed as area of the immunoprecipitate) of 107 mm^2 (± 25). The corresponding value for the 8 patients undergoing a pre-injection of contrast material was 37 mm^2 (± 63) ($p < .02$). During periods 2 and 3 the values for trypsin in the contrast group were 100.1 mm^2 (± 20.7) and 95.2 mm^2 (± 19.8). For the non-contrast group the corresponding values were 104.4 mm^2 (± 43.7) and 95.8 mm^2 (± 39.8). Neither of these groups was significantly different ($p > 0.5$) (Fig. 2).

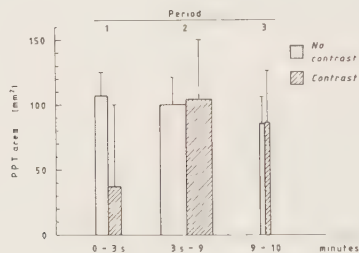


Fig. 2. Bar graph representation of radialimmunodiffusion analysis for human cathodal trypsin in undiluted specimens of pancreas juice.

Findings for PSTI and PE were similar and are summarized together with the above data in Table 1.

Table 1.

RADIALIMMUNODIFFUSION

Trypsin (mm ² of PPT)			PSTI (mm ² of PPT)			PE (mm ² of PPT)		
	NC	C		NC	C		NC	C
Period 1	107.0 (± 25)	37.0 (± 63)		123.6 (± 19.6)	21.7 (± 4.0)		103 (± 18)	39 (± 18)
Period 2	100.1 (± 20.7)	104.4 (± 43.7)		94.7 (± 22.4)	72.9 (± 29.2)		98 (± 26.1)	93 (± 35.3)
Period 3	95.2 (± 19.8)	95.8 (± 39.8)		84.2 (± 16.3)	73.8 (± 32.3)		96.7 (± 241.1)	93.8 (± 34.4)

* no significant $p > 0.2$

** probably not significant $0.2 > p > 0.1$

*** significant $p < 0.01$

Values expressed in area of precipitate for Trypsin, Pancreatic Elastase (PE), and Pancreatic Secretory Trypsin Inhibitor (PSTI) determined by radialimmunodiffusion of undiluted specimens of pancreas juice. C = specimens from patients undergoing contrast pre-injection. NC = specimens from patients not undergoing a contrast pre-injection.

Periods are times in relation to i.v. stimulation with secretin/CCK-PZ (10/Kg to a maximum of 75 U)
See text.

In the second experiment, increasing amounts of contrast material were found to have no effect on the size or the character of the immunoprecipitates.

Radioimmunoassay studies of the period 2 samples can be seen in Table II. Virtually no difference exists within group for the values of PE and PSTI. Although there is an apparent difference for trypsin, it is not statistically significant.

Amylase, which was determined enzymatically, likewise was not found to be statistically different between the two groups. It was 300 $\mu\text{kat/l}$ (± 192) for the contrast group and 485 $\mu\text{kat/l}$ (± 207) in the non-contrast group ($p > 0.2$).

Table II.

RADIOIMMUNOASSAY

Period 2 samples

<u>Trypsin ($\mu\text{g/l} \times 10^4$)</u>		<u>PSTI ($\mu\text{g/l} \times 500$)</u>		<u>PE ($\mu\text{g/l} \times 10^4$)</u>	
<u>NC</u>	<u>C</u>	<u>NC</u>	<u>C</u>	<u>NC</u>	<u>C</u>
22.5 (± 18.9)	34.7 (± 16.5)	12.2 (± 1.9)	14.9 (± 2.2)	18.2 (± 11.4)	18.8 (± 6.7)
(.5 > p > .1)		(p > 0.5)		(p > 0.5)	

Values for trypsin, Pancreatic Secretory Trypsin Inhibitor (PSTI) and Pancreatic Elastase (PE) determined using RIA on diluted pancreas juice. C = contrast pre-injection/NC = no contrast pre-injection.

Only period 2 collections (3.5 - 9 min. post i.v. stimulation with secretin/CCK-PZ) were assayed (see text).

DISCUSSION

Previous authors have clearly demonstrated alterations in pancreas juice secreted by patients having chronic pancreatitis and pancreatic carcinoma (7,8,9,10,11). Changes are found in volume, bicarbonate concentrations, hexosamine, calcium and total protein or "enzyme" content of the juice. Allan and White explored this latter observation using a combination of isoelectric focusing and sophisticated densitometric computer assisted scanning techniques. They clearly demonstrated a selective depression of materials in the cationic spectrum in patients having pancreatic carcinoma, but not periampullary carcinoma. However, they could not completely identify these bands (11).

The study of proteins in pure pancreas juice as they relate to different pancreatic diseases seems to be an important investigational area which has not been completely explored. Endoscopy offers a ready means of collecting pancreas juice for experimental study. There have been several major problems concerning its routine use for this purpose during clinical procedures: if contrast media is avoided, selective cannulation of the pancreas duct is cumbersome and time consuming, necessitating ERP be performed as a separate experimental procedure so as not to interfere with routine clinical studies; the effect of radiocontrast material on pancreas juice has not been determined; the effect of contrast material on the quantitation of proteins in the juice is unknown; and an optimal period of collecting pancreas juice following selective cannulation of the main pancreatic duct has not been defined.

In this study, when contrast material was used for the ERP, less than 15 min were added to the procedure. Subjectively the protocol did not add significantly to patient discomfort, and there were no cases of post-operative pancreatitis. In no case was the requisite morphological study compromised by the collection of pancreas juice.

Eddeland and Wehlin previously demonstrated peak secretion of both volume and protein 4 to 5 min after an intravenous bolus injection of secretin and CCK-PZ (1). Using these parameters,

we were able to obtain peak protein output in samples collected 3.5 to 9 min after a similar stimulation (Fig. 2). However, despite a "wash-out" period of 3.5 min, some roentgenographically detectable contrast media was present in 80 % of the samples.

We found no effect of the contrast media on the quantitative performance of our radioimmunoassays or on the qualitative or quantitative behavior of immunoprecipitates formed in agarose during radial-immunodiffusion. Also, there was no influence of the contrast media on the enzymatic technique used to quantitate α -amylase. Therefore, these widely used analytic methods are valid even when some contrast media is present in the samples.

Pancreas juice which was inactive against the trypsin substrate BAPNA remained inactive following incubation with increasing amounts of contrast media at room temperature for at least 30 min. Therefore, juice collected in this manner can be used with confidence during bench work analysis.

Finally, samples of trypsin-inactive pancreas juice collected at ERP were stored for periods up to 1 year at -20°C . These samples remained inactive despite contamination with radio-contrast media. Thus long term storage of samples is possible which allows for large batch analysis of samples minimizing interbatch variation.

In summary, we have described a protocol for the collection of pure pancreas juice for experimental study. Collection can be performed during clinical testing as it does not interfere with the requisite morphological studies. We have also demonstrated that contamination of the juice with radio-contrast media does not seem to affect either the juice itself or several popular analytic techniques.

ACKNOWLEDGEMENTS

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This investigation was supported by grants from the Swedish Medical Research Council (project no. B83-17X-03910-11B), the Medical Faculty, University of Lund, the Foundation of Malmö General Hospital against Cancer, Torsten and Ragnar Söderberg Foundation and University of Michigan, Department of Surgery.

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PANCREATIC SECRETORY TRYPSIN INHIBITOR, CATHODAL TRYPSIN AND PANCREATIC ELASTASE IN PANCREAS JUICE COLLECTED AT ERCP^{*}

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Pancreas juice collected from 61 patient volunteers by an endoscopic method was quantitatively analyzed for cationic trypsin, PSTI, pancreatic elastase and α -amylase. Patients were grouped and analyzed as cigarette smokers vs. non-smokers, over 65 vs. under 65 years of age, and according to diagnosis.

Patients with pancreatitis and extra-pancreatic neoplasms had decreased levels of trypsin and PSTI compared to patients without demonstrable pancreatic or neoplastic disease.

Patients with pancreatic carcinoma were found to have a markedly decreased concentration of PSTI and decreased volumes of pancreas juice produced in response to cholecystokinin/secretin intravenous stimulation when compared to all other diagnostic categories.

^{*} Read before The World Congress of Gastroenterology, Stockholm June 1982

The human exocrine pancreas produces about 10 grams of protein per liter of fluid each day. Its protein products are relatively well defined and consist of at least 25 constituents as determined by isoelectric focusing (IEF) (1).

Previous authors have clearly shown a decrease in protein or "enzyme" output in patients having chronic pancreatitis and pancreatic carcinoma. To date White and Allan (2) and more recently, Rinderknecht, *et al* (3) have attempted to define changes in specific protein products as they relate to pancreatic diseases. They have described specific changes associated with pancreatic carcinoma.

Specific immunochemical methods of analysis are available in our laboratory for several human exocrine pancreatic proteins.

The purpose of this work was to analyze pure pancreas juice collected by an endoscopic method from patient volunteers for cathodal trypsin immunoreactivity, Pancreatic Secretory Trypsin Inhibitor (PSTI) and pancreatic elastase.

MATERIALS AND METHODS

Antisera: Specific antisera to human cationic trypsin (4), human pancreatic elastase II (5), and human PSTI (6) are available in our laboratory. Analyses were made using RIA on appropriately diluted samples. All were performed in duplicate. PSTI was also analyzed after incubation of the juice sample with Trasylol (7).

α -Amylase was analyzed using the Phadebas Enzymatic Technique as used at the Malmö General Hospital, Department of Clinical Chemistry.

Pancreas juice was collected from patient volunteers referred to the endoscopic unit for routine Endoscopic Retrograde Cholecho-pancreatography (ERCP) for a variety of complaints. All gave informed consent. The method was as described by us elsewhere (8). Briefly, the pancreas juice was collected following intravenous stimulation with cholecystokinin-pancreozymin (CCK) and secretin (1 CU/Kg to a maximum of 75 CU of each). (Both produced by KABI, Sweden). Collection took place over the next 9 minutes. The initial 3.5 minute collection was discarded. Pancreas juice

collected over the remaining 5.5 minutes was then quantitated, divided into aliquots and stored until analyzed.

Patient volunteers: 84 patients were entered into the collection protocol over a 1½ year period. Pancreas juice was collected successfully in 71. Top of these 71 patients were rejected due to experimental design error, contamination of the pancreas juice sample with bile or incomplete medical work-up. The 61 remaining patients could be classified into one of six diagnostic groups as follows:

Normal (n=9) no demonstrable pancreatoco-biliary disease
or known malignancy

Pancreatic carcinoma (n=9)

Other carcinoma (n=8) *

Hepatobiliary disease (n=32) *

Pancreatitis (n=6): chronic (3) or recurrent acute
acute attacks (3)

* 3 of these patients were included in both categories.

Data was also grouped for analysis as follows:

A. Cigarette smokers vs. non-smokers:

Smokers (n=39): smoked cigarettes more
than 1 pack-year and ad-
mitted to continued use
at the time of the exami-
nation

Non-smokers (n=18): never smoked cigarettes,
or smoked less than 1
pack-year but had not
smoked within 3 years of
the examination

Poor or uncertain history (n=4): not used for this part
of the study

B. According to age:

≥ 65 years of age (n=20)

< 65 years of age (n=41)

Statistics were performed using Student's t test.

RESULTS

Cigarette smokers vs. non-smokers: No significant differences or trends were apparent between cigarette smokers and non-smokers (Fig. 1).

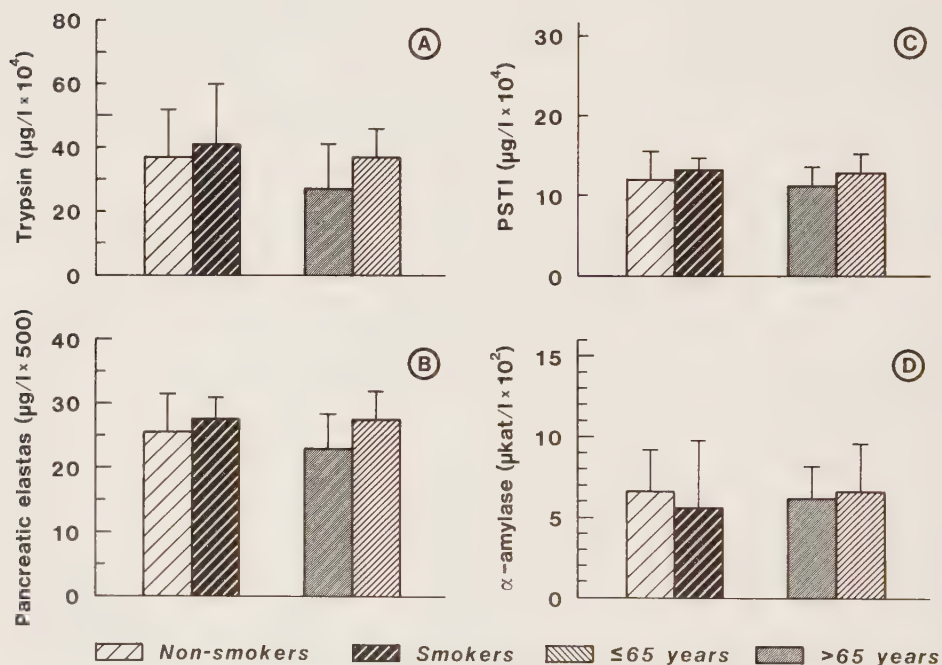


Fig. 1. Concentrations of trypsin, pancreatic elastase, PSTI and α -amylase in pancreas juice from humans categorized on the basis of age and smoking history (see text for details).

Age groups: No significant differences or trends were apparent between patients 65 years or older and those less than 65 years of age (Fig. 1).

Diagnostic groups: All groups were compared to the "control" or "no known disease" group. PSTI, PE and trypsin were all decreased in patients having hepatobiliary disease although the decrease in PE was not statistically significant (Fig. 2 A,B,C). No trends were apparent for α -amylase in any category (Fig. 2 D).

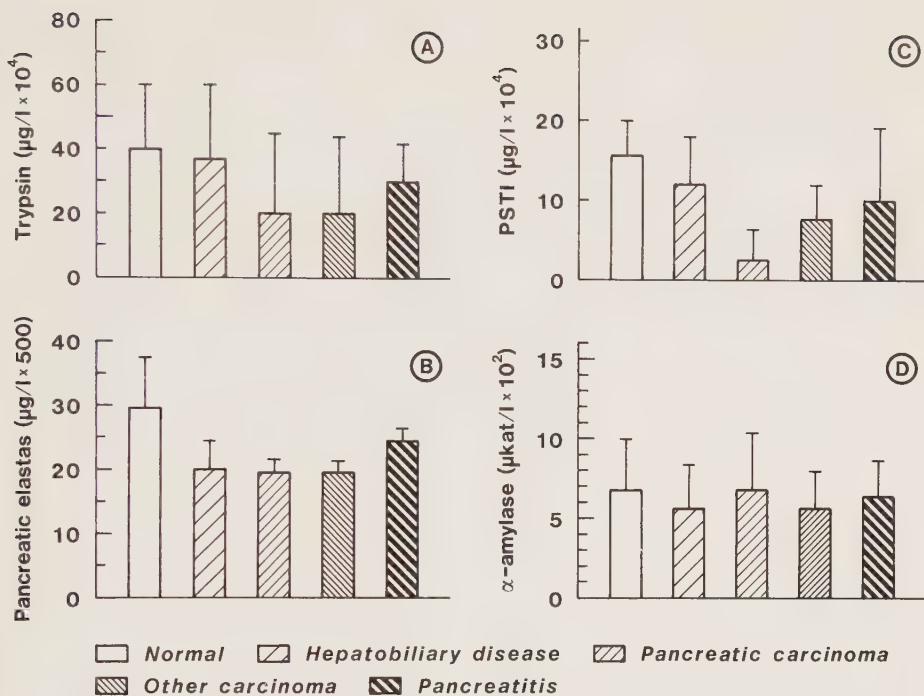


Fig. 2. Concentrations of trypsin, pancreatic elastase, PSTI and α -amylase in pancreas juice from humans categorized according to diagnosis (see text for details).

Trypsin was significantly decreased in the pancreas juice of patients having pancreatic carcinoma ($p=.02$), other carcinoma ($p < .02$) and pancreatitis ($p=.05$) (Fig. 2 A). PSTI was decreased but not significantly in pancreatitis and markedly decreased in patients having other carcinomas ($p<.05$) and pancreatic carcinoma ($p<.001$) (Fig. 2 C).

The PSTI concentration did not increase significantly in any sample after incubation with Trasylol. The volumes of pancreas juice collected showed considerable variability. The values are presented in a dot graph (Fig. 3) to demonstrate the trends.

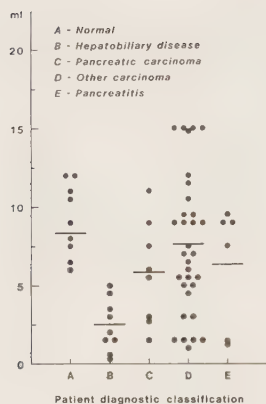


Fig. 3. Volume of pancreas juice collected by direct duct cannulation of the gland from 3.5 to 9 min post intravenous secretin/cholecystokinin stimulation (see text for details).

DISCUSSION

Balldin *et al* recently reported increased levels of trypsin immunoreactivity in the peripheral circulation of smokers in response to secretin stimulation (7). If this were the result of an increased synthesis or release in response to secretin, increased concentrations may be anticipated in the pancreas juice.

No affect of cigarette smoking was found on the concentrations of the proteins investigated. Likewise no quantitative differences were apparent when the samples were analyzed on the basis of age.

Others have reported that diseases of the pancreas result in a decreased output of pancreatic exocrine secretions (8,9,10). Allan and White described selective decreases in some proteins of patients having pancreatic carcinoma (2), but not in patients having periampullary carcinoma. In their study IEF with sophisticated densitometric scanning was used. Materials in the cationic spectrum were studied, however, they have not completely identified the depressed components. Rinderknecht *et al* in work independent from ours have recently described a decrease in digestive enzymes and trypsin inhibitor in patients with pancreatic carcinoma. Using a different technique for analysis,

they found the trypsin inhibitor to be the only protein which was consistently lower in concentration than the lowest recorded 'normal' (3).

We have found significantly decreased concentrations of both trypsin and PSTI in patients having hepatobiliary disease, pancreatitis and pancreatic carcinoma. PSTI concentrations were of special interest, being decreased far more in patients with pancreatic carcinoma than in any of the other diagnostic categories. The decreased levels of PSTI may be due to decreased production, decreased secretion or consumption due to local pancreatic inflammation in the area of the tumor.

The exact mechanism remains to be determined, however, we do know from previous work that our anti-PSTI serum detects only free PSTI (11). We have also demonstrated that Trasylol is capable of rapidly displacing PSTI from a complex with trypsin *in vitro*. When Trasylol was placed in samples having low PSTI concentrations either no or minimal increases were noted. Therefore, it seems unlikely that PSTI is decreased in patients with pancreatic carcinoma on the basis of local complexation of the inhibitor. Whether this finding has diagnostic potential remains to be seen.

In addition, we have demonstrated that extra-pancreatic carcinoma can result in decreased concentrations of trypsin and PSTI in pancreas juice. It is possible that systemic influences of these carcinomas on protein synthesis account for this, however, pancreatic elastase and amylase were not similarly decreased.

Finally, the volume of pancreas juice produced was significantly less in patients having pancreatic carcinoma than with any of the other disease processes. As most of the carcinomas used in this study were relatively advanced, ductal obstruction cannot be ruled out as a major contributing cause for this finding.

ACKNOWLEDGEMENTS

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IMMUNOCYTOCHEMICAL DISTRIBUTION OF TRYPSINOGEN AND PANCREATIC SECRETORY TRYPSIN INHIBITOR (PSTI) IN NORMAL AND NEOPLASTIC TISSUES IN MAN

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Immunoreactive trypsinogen and PSTI both showed an identical distribution in acinar cells of "normal" human exocrine pancreas tissues. Ductal adenocarcinoma tissue and pancreatic undifferentiated carcinoma contained neither antigen. Scattered "normal" looking cells in the border area between normal and neoplastic tissue of both types of tumor stained positively for trypsinogen as well as for PSTI.

Key words: Pancreas; trypsin; trypsin inhibitors

The localization of very few pancreatic exocrine proteins has been reported to date in human pancreas (1,2). The distribution of these antigens may be important in determining the cells of origin of exocrine pancreatic tumors in man (3,4).

Rat pancreatic carcinoma, like that of most mammals, is believed to be of acinar cell origin (5,6,7). The vast majority of human pancreatic carcinomas are generally thought to be of ductal origin. This belief is based on the presence histologically of tubular structures which define ductal elements (3,8). However, Bockman has recently presented evidence that such structures may represent normal variants or inducible variants of acinar cells, a process he and Reddy (3,9,10) refer to as de-differentiation of acinar cells. By de-differentiation they mean a loss of secretory ability and height of acinar cells, and the resultant formation of tubular elements. Therefore, the cells of origin of human pancreatic carcinoma remain in question.

Hansen *et al.* recently studied the distribution of trypsinogen, chymotrypsinogen A, α -amylase, lipase, ribonuclease and carboxypeptidase A in normal and neoplastic rat pancreas. Using an immunofluorescence technique they found staining for all six enzymes in all acinar and neoplastic cells (4).

If human pancreatic carcinoma represents acinar cell de-differentiation, secretory antigens may be present in the neoplastic cells, much as described by Hansen in the rat.

The purpose of this work was to study the distribution of exocrine pancreatic proteins in "normal" and neoplastic human pancreatic tissue. We have chosen to study trypsinogen, the key protease of pancreas juice, and a second antigen, the low molecular weight pancreatic secretory trypsin inhibitor of Kazal (PSTI). While it has been generally assumed that PSTI is an acinar cell secretory product, its distribution in the pancreas has not been previously demonstrated.

MATERIALS AND METHODS

Human specimens: Human material was kindly provided by the Pathology Departments of the University of Lund at the Malmö General and Lund University Hospitals. Specimens were from four patients with chronic pancreatitis, six patients with well differentiated pancreatic carcinoma classified to be of "ductal" origin and two with undifferentiated pancreatic carcinoma.

Antiserum: Human cathodal trypsin and PSTI were isolated, as previously described, from human pancreas juice (11,12). Rabbit anti-human cathodal trypsin and goat anti-human PSTI were prepared using Freund's complete adjuvant as described earlier (12,13).

Immunohistochemical method: The antigens were demonstrated using an immunoperoxidase procedure, according to Mason *et al.* (14). Background staining was reduced using pepsin digestion according to Reading (15). Only one antigen was stained for on any one slide. Alternate slides were stained with trypsin or PSTI to allow comparison of staining in the same cells or areas of tissue.

Each antiserum was applied in dilutions ranging from 1:100 to 1:1000 or 1:2000. After washing off the 3, 3'-diamino-benzidine tetrahydrochloride, which develops the brown color of positive antigen staining, a standard hematoxylin counter stain was applied.

Controls were performed with each new staining using the appropriate antigen absorbed antiserum in a dilution of 1:100. Final preparations were dehydrated and permanently preserved in a synthetic medium (Pertex, Bethlehem Trading Ltd., U.K.).

RESULTS

Acinar cells in "normal" areas of the gland stained positively for both trypsinogen and PSTI (Figs. 1-2). Differences in the intensity of staining were noted. In some sections peri-islet areas contained relatively darker staining than other parts of the gland for both antigens, however, this finding was not consistent. Islets served as internal background controls as they did not stain for either antigen.

Serial sections demonstrated that the same cells were positive for both trypsinogen and PSTI. Ductal adenocarcinoma tissue was devoid of both antigens in all specimens studied (Fig. 3). Areas on the border between normal and neoplastic tissues of the ductal type contained atypical formations and pseudoacinar tissue. Some of these elements stained positively for trypsinogen or PSTI, but some contained neither (Fig. 4). The two examples of undifferentiated pancreatic carcinoma contained neither antigen, while normal appearing areas of tissue on the same slide contained both. Scattered cells in the border-area between normal and neoplastic tissues also stained positively for both antigens (Figs. 5-6).

DISCUSSION

We have shown that immunoreactive trypsinogen and PSTI are each widely distributed throughout "normal" exocrine pancreas tissue in man. This agrees with the findings of others in the pancreas of lower animals, that trypsin is produced throughout all the acinar cells of the gland (16,17,18,19). This study also represents the first demonstration of the acinar cell origin of PSTI. Its generalized distribution in exocrine tissue supports the belief of Laskowski Jr and others that tissues which produce proteases also produce low molecular weight specific inhibitors as a means of local protection (20).

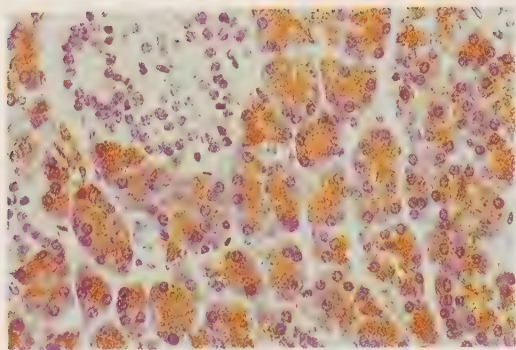
While the nature of the material for this study precludes the use of truly "normal" tissues, no differences in the qualitative appearance of the acinar cell content of trypsinogen or PSTI

were evident in normal areas of glands removed for either pancreatitis or pancreatic carcinoma.

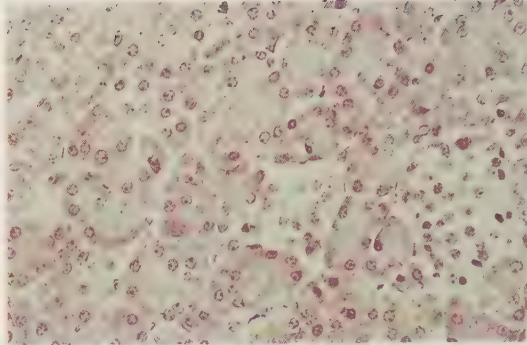
In 1975 Malaisse-Lagae *et al.* (21) and, from the same laboratories in 1979, Bendayan and Ito (16) reported findings of acinar cell topographical differentiation using the rat pancreas. These workers used an immunofluorescence technique to study α -amylase, lipase, chymotrypsin, and trypsinogen and chymotrypsin A, α -amylase, carboxypeptidases A and B, DNase, elastase, lipase and RNase A, respectively. They defined a greater intensity of exocrine proteins in the acinar cells immediately adjacent to islets, as opposed to cells distributed throughout the rest of the gland on the basis of increased immunofluorescence. Empirically we have also observed what appear to be areas of increased staining about the islets, especially for PSTI. However, this finding was inconsistent and as this technique is not quantitative, may be factitious.

Bockman has recently described electron and light microscopic studies of a rat pancreas tumor model (5,6). He described "transitional stages" between acinar and tubular cell complexes. His overall impression was that this form of pancreatic carcinoma represented an acinar cell de-differentiation as opposed to ductal cell tumor invasion and destruction. Others have reported similar findings in guinea pig (9) and hamster (22) models.

To date, only one report exists in the literature which describes the distribution or presence of pancreatic exocrine proteins in pancreatic tumor tissues. Hansen *et al.* (4) used a transplantable acinar cell rat tumor and, using an immunofluorescence technique stained for α -amylase, lipase, carboxypeptidase A, chymotrypsinogen, trypsinogen and RNase. All six enzymes were present in all cells of both normal and tumor tissue. We did not find positive staining for trypsinogen or PSTI in either the well differentiated or the undifferentiated tumors. We did find the presence of bizarre structures, some of which contained antigen, on the border between tumor and relatively normal areas. These structures may represent transitional elements or remaining parts of normal tissue.



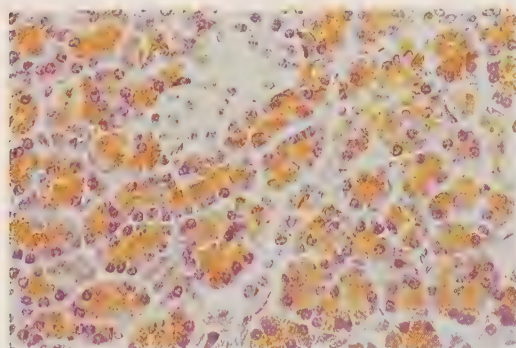
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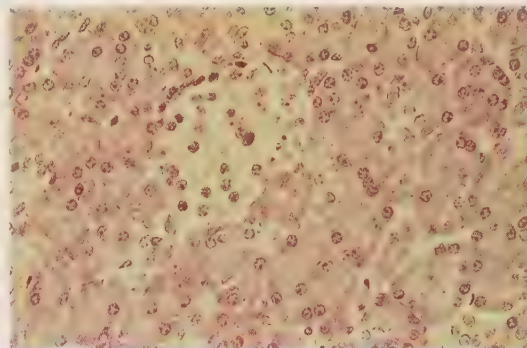
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Fig. 1. "Normal" area of pancreatic tissue from a patient with chronic pancreatitis incubated with a) rabbit antiserum against human cationic trypsin and b) the same

antiserum after absorption with trypsin. a) Shows positive brown staining of trypsin in the acinar cells.



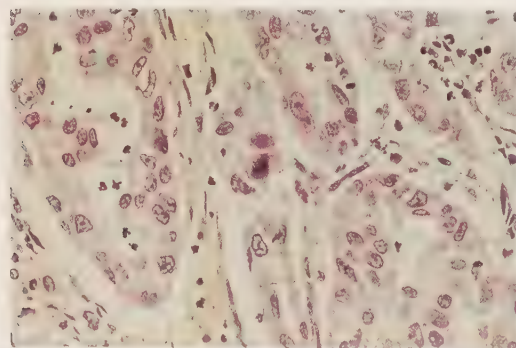
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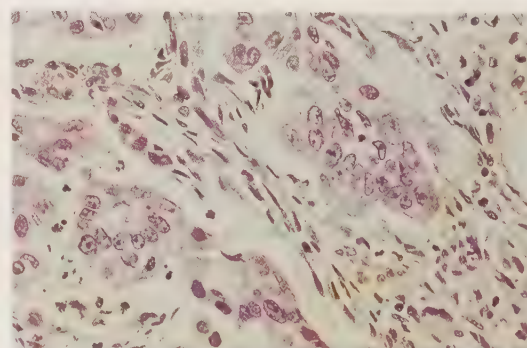
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Fig. 2. "Normal" pancreatic tissue (see text fig. 1) incubated with a) rabbit antiserum against human pancreatic secretory trypsin inhibitor (PSTI) and b) the same

antiserum after absorption with PSTI. a) Shows positive brown staining of PSTI in the acinar cells.



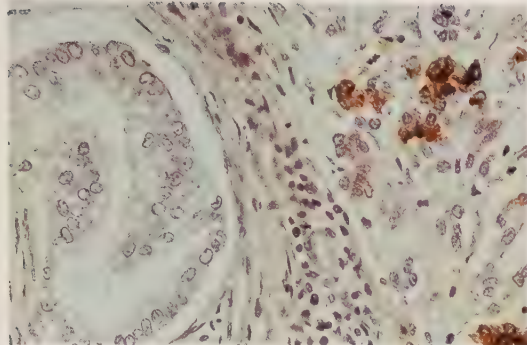
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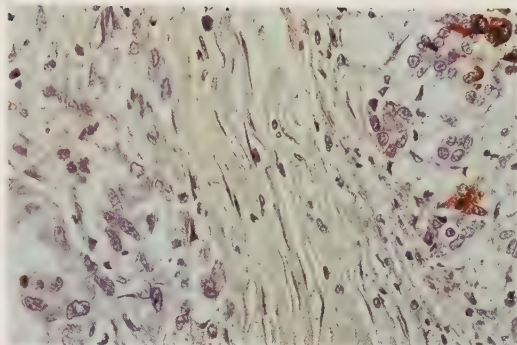
Fig. 3. Central area of ductal pancreatic carcinoma incubated with a) antiserum against trypsin and b) antiserum against

PSTI. No positive brown staining is seen in the tissue.



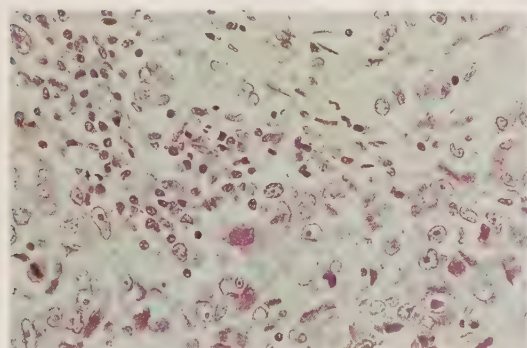
a

Fig. 4. Border area between normal and neoplastic tissue of the ductal type incubated with a) antiserum against trypsin and b)



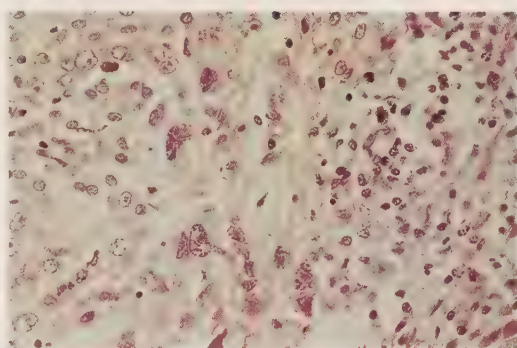
b

antiserum against PSTI. Positive brown staining of trypsin and PSTI, respectively, is seen in scattered cells.



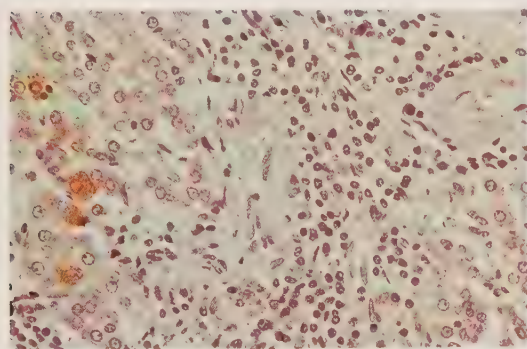
a

Fig. 5. Central area of undifferentiated pancreatic carcinoma incubated with a) antiserum against trypsin and b) antiserum



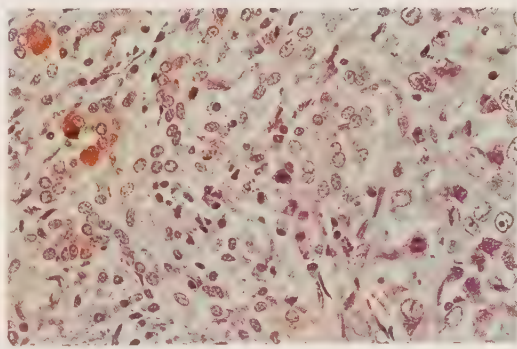
b

against PSTI. No positive brown staining is seen in the tissue.



a

Fig. 6. Border area between normal and neoplastic tissue of the undifferentiated type incubated with a) antiserum against trypsin and b) antiserum against PSTI.



b

Positive brown staining of trypsin and PSTI, respectively, is seen in scattered cells.

While the material used in this initial study is small, the consistent lack of secretory products in tumor tissue is striking. Our findings support the concept that human pancreatic tumors are of ductal origin. Further work is obviously needed to verify these findings. A larger population of samples, including ultra-structure localization of the proteins using protein-A gold complex, are currently being investigated in our laboratory.

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